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## CONTENTS

NUMBER 1, SEPTEMBER, 1948

I. The Toxicity of Some Zirconium and Thorium Salts in Rats. L. T. McClinton and Jack Schubert . . . . .	1
II. The Potentiating Effect of Antihistaminic Drugs Upon the Sedative Action of Barbiturates. Charles A. Winter . . . . .	7
III. Studies on Hydrocarbon-Epinephrine Induced Ventricular Fibrillation. Solomon Garb and Maynard B. Chenoweth . . . . .	12
IV. The Inhibition of the Cholinesterase of Rat Brain by Methadon. George S. Eadie, Frederick Bernheim and Dorothea B. Fitzgerald. . . . .	19
V. The Estimation of Acetanilide and Its Metabolic Products, Aniline, N-Acetyl p-Aminophenol and p-Aminophenol (Free and Total Conjugated) in Biological Fluids and Tissues. Bernard B. Brodie and Julius Axelrod	22
VI. The Fate of Acetanilide in Man. Bernard B. Brodie and Julius Axelrod	29
VII. Comparison of Ouabain with Strophanthidin-3-Acetate by Intravenous Injection in Man Harry Gold, Walter Modell, Nathaniel T. Kwit, Samuel J. Shane, Conrado Dayrit, Milton L. Kramer, William Zahm and Harold L. Otto	39
VIII. Studies on Purified Digitalis Glycosides. VI Thevetin, a Glycoside with Unusual Speed of Action in Man. Walter Modell, Nathaniel T. Kwit, Conrado Dayrit, Samuel J. Shane, William Zahm, Milton L. Kramer and Harry Gold	44
IX. A Study of the Pharmacological Relationship Between Digitoxin and Histamine A. Sjoerdema and E. Kun	53
X. Synergistic Effect of Chlorguanide and Sulfadiazine against Plasmodium gallinaceum in the Chick. Joseph Greenberg, Beverly L. Boyd and Edward S. Josephson	60
XI. A Study on the Comparative Toxic Effects of Citric Acid and Its Sodium Salts Charles M. Gruber, Jr. and William A. Halbeisen	65
XII. An Ultraviolet Spectrophotometric Procedure for the Determination of Barbiturates. Leo R. Goldbaum	68
XIII. The Effect on the Pain Threshold of N-Acetyl p-Aminophenol, a Product Derived in the Body from Acetanilide. Frederick B. Flinn and Bernard B. Brodie .. .	76
XIV. On the Mechanism of the Convulsant Action of Strychnine; the Lack of Atropine Antagonism. W. Clarke Wescoe and Ray E. Green	78

NUMBER 2, OCTOBER, 1948

XV. Naphthoquinone Antimalarials XVIII. Metabolic Oxidation Products. Louis F. Fieser, Frederic C. Chang, William G. Dauben, Hans Heymann and Arnold M. Seligman	85
XVI. Naphthoquinone Antimalarials. XIX Antirespiratory Study of Protein Binding Hans Heymann and Louis F. Fieser	97
XVII. Naphthoquinone Antimalarials. XX Metabolic Degradation. Louis F. Fieser, Hans Heymann and Arnold M. Seligman	112
XVIII. Thyroxine-Like Activity of Chloro- and Bromo-thyronine Derivatives. C. P. Leblond and B. Grad	125
XIX. Enzymic Hydrolysis of Morphine Esters. Sidney Ellis . . . . .	130
XX. Further Studies on the Antifibrillatory Action of Coronary Dilator Drug K. I. Melville . . . . .	. . . . .

XXI. A Pharmacologic Study of Ortho-Methoxy-beta-Phenylisopropyl Methylamine Hydrochloride, and Thirteen Related Methoxy Analogues. Boyd E. Graham and Marvin H. Kuizenga.....	150
XXII. The Absorption and Elimination of Caronamide (4'-Carboxyphenylmethanesulfonanilide). Karl H. Beyer, Elizabeth K. Tillson, Horace F. Russo, Grace S. Schuchardt and S. Richard Gass.....	167
XXIII. A Comparison of One Dose Per Day with Six Doses Per Day of Quinine in the Suppression of Lophurae Malaria in the Duck. Earl H. Dearborn....	178
XXIV. The Influence of Lung Distension Upon the Response of the Bronchioles to Epinephrine and to Histamine. K. I. Melville and H. Caplan.....	182
XXV. The Comparative Pharmacology of p-Fluorophenethylamine and p-Fluorophenethyl-Methylamine. David F. Marsh.....	192
XXVI. Pharmacology of a New Antihistamine, $\beta$ -Pyrrolidine-Ethyl-Phenothiazine (Pyrrolazote). Milton J. Vander Brook, Kenneth J. Olson, Marilyn T. Richmond and Marvin H. Kuizenga.....	197
XXVII. A Preliminary Pharmacological Investigation of the Tannin Obtained from Pinus Caribaea, Morelet. Frank W. Bope, Elizabeth M. Cranston and Ole Gisvold .....	209
XXVIII. The Relationship of Cholinesterase Inhibiting Activity to the Toxicity of Some Organic Phosphorus Compounds. D. H. Walker Jones, Jr., Bertram J. Meyer and Leonard Karel.....	215

## NUMBER 3, NOVEMBER, 1948

XXIX. The Effect of Adrenergic Blocking Agents on the Vasoconstriction Produced by Acute Oxygen Lack. David F. Marsh and Edward J. Van Liere.....	221
XXX. The Comparative Pharmacology of the Isomeric Heptylamines. David F. Marsh.....	225
XXXI. Antibiotic Lactones and Synthetic Analogs. I. Cardiotoxic Effects on the Isolated Frog Heart. Nicholas J. Giarman.....	232
XXXII. Influence of Tetraethylammonium on Responses of Isolated Intestine to Angiotonin and Other Substances. Dean A. Collins.....	244
XXXIII. A Neurological Syndrome Induced by Administration of Some Chlorinated Tertiary Amines. A. Goldin, H. A. Noe, B. H. Landing, D. M. Shapiro and B. Goldberg.....	249
XXXIV. Concerning the Inability of 2,3 Dithiopropanol (BAL) to Prevent or to Modify the Acute Renal Injury Induced by Uranium Nitrate. Wm. de B. MacNider, Jesse C. Trott, Jr. and Margaret D. Bruce.....	262
XXXV. The Effect of Cardiac Glycosides on the Respiration of Cardiac Muscle. Murray Finkelstein and Oscar Bodansky.....	274
XXXVI. Studies on the Laxative Activity of Triphenylmethane Derivatives. I. Relationship between Structure and Activity of Phenolphthalein Congeners. S. Loewe.....	288
XXXVII. A Comparison of Nine Local Anesthetics. H. S. Hamilton, B. A. Westfall and J. K. W. Ferguson .....	299
XXXVIII. Biological Assay of Thyroid Activity. D. E. Hutcheon .....	308
XXXIX. Anesthesia. XXXI. A Study of Cyclic and Noncyclic Hydrocarbons on Cardiac Automaticity. John C. Krantz, Jr., C. Jelleff Carr and James F. Vitcha.....	315
XL. The Role of Arterial Pressure in the Induction of Idioventricular Rhythms under Cyclopropane Anesthesia. Gordon K. Moe, S. Donald Malton, Barbara R. Rennick and Walter A. Freyburger.....	319
XLI. The Evaluation of the Analgesic Action of Methadon Isomers and Other Analgesics by a New Rapid Screening Method. Raymond L. Cahen, Herbert J. Epstein and Christie S. Krementz.....	328

NUMBER 4, DECEMBER, 1948

XLII. Adrenergic Blocking Drugs. II. Antagonism of Histamine and Epinephrine with N-(2-haloalkyl)-1-naphthalene-methylamine Derivatives. Earl R. Loew and Audrey Micetich . . . . .	339
XLIII. Adrenergic Blocking Drugs. III. Effects of Two Halogenated Ethylamines on Pressor Responses to Epinephrine, Nicotine and Adrenergic Nervous Reflexes. Clem A. Stone and Earl R. Loew . . . . .	350
XLIV. The Fate of Procaine in Man Following Its Intravenous Administration and Methods for the Estimation of Procaine and Diethylaminoethanol. Bernard B. Brodie, Philip A. Lief and Raymond Poet . . . . .	359
XLV. Protective Action of Combinations of Diphenhydramine and Aminophylline in Fatal Bronchospasm in Guinea Pigs Due to Mecholyl Aerosol. W. E. Hambourger, Homer B. Freese, Martin M. Winbury and Patricia M. Michiels.. . . . .	367
XLVI. Antagonism of Rutin to Capillary Effects of Sodium Bisulfite and Procaine. R. K. Richards and K. E. Kueter. . . . .	372
XLVII. Hindlimb Reflexes of Chronic Spinal Dogs During Cycles of Addiction to Morphine and Methadon Abraham Wikler, Senior Surgeon, U.S.P.H.S. and Karl Frank, Biophysicist. . . . .	382
XLVIII. Bromal Hydrate and Chloral Hydrate: A Pharmacological Contrast and Its Chemical Basis. Thomas C. Butler . . . . .	401
XLIX. The Tracheal Chain. II. The Anaphylactic Guinea Pig Trachea and Its Response to Antihistamine and Bronchodilator Drugs. Julio C. Castillo . . . . .	412
L. Comparative Actions of Certain Compounds Like Alpha-Fagarine. Gordon A. Alles and Charles H. Ellis . . . . .	416
LI. The Pharmacological Activity of 1-Phenyl-2-Butylamine. David F. Marsh . . . . .	426
LII. Studies on the Toxicity, Distribution and Excretion of Emetine. Abraham I. Gimble, Clarke Davison and Paul K. Smith . . . . .	431
LIII. Experimental Heart Failure Caused by Central Nervous System Depressants and Local Anesthetics. Albert Wollenberger and Otto Krayer . . . . .	439
LIV. Action of Narcotics and Local Anesthetics on the Respiration of Heart Muscle. Albert Wollenberger . . . . .	444
LV. An Investigation of the Nature and Extent of the Binding of Oxophenarsine (Mapharsen) by the Red Blood Cell of the Rabbit in Vitro. I. Adsorption on the Cell Surface. Lewis D. Fink and Harold N. Wright . . . . .	455
LVI. An Investigation of the Nature and Extent of the Binding of Oxophenarsine (Mapharsen) by the Red Blood Cell of the Rabbit in Vitro: II. Factors Other Than Adsorption. Lewis D. Fink and Harold N. Wright . . . . .	462



# THE TOXICITY OF SOME ZIRCONIUM AND THORIUM SALTS IN RATS<sup>1</sup>

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Received for publication February 20, 1948

In connection with investigations directed toward finding a treatment for radioelement poisoning (1) a study was made of the toxicity of several metals, including zirconium and thorium. In the experiments reported here the object was to study the lethal action of some salts of zirconium and thorium and to note any chronic intoxication.

**EXPERIMENTAL PROCEDURES.** The rats used were 200 gram white females of the Sprague-Dawley strain. All solutions were administered by intraperitoneal injection. The solutions did not appear to cause any pain or discomfort to the animals. In all cases suitable control animals were kept. The injected groups of control animals received injections of solutions similar to the test solutions except for the absence of the specific element under investigation. All animals were dosed on the basis of weight for not more than 20 hours in the acute studies and not more than 8 days in the sub-acute studies (see tables 1 and 2 for details). They were weighed weekly for six months subsequently. Histologic examination was made of the organs of three of the rats which had received high concentrations of zirconium as the citrate salt. Total leucocyte and differential counts, hemoglobin concentrations and the clotting times of the blood were determined on two rats three days after they had received their last intraperitoneal injection of zirconium citrate.

**Preparation of solutions.** In the preparation of the citrate salt of zirconium it was desired to obtain a maximum zirconium concentration with a minimum of sodium and citric acid. Further, it was deemed necessary that the solution be stable to wide changes in temperature and pH as well as yielding no precipitate upon the addition of phosphate ions. Such a solution is prepared as follows:

Dissolve 14.92 grams (0.071 M) of citric acid monohydrate in 250 cc. of water. To the boiling solution slowly add 75 cc. zirconyl chloride solution containing about 125-130 mgm. of zirconium per cc. Boil for ten minutes with constant stirring (avoid splatter) while keeping the volume nearly constant by addition of water. Cool to room temperature and filter through quantitative filter paper on a Büchner funnel. After washing the precipitate with 100 cc. of water it is suspended in 200 cc. of water and refiltered through fresh filter paper. Wash precipitate with 100 cc. of water. Suspend the precipitate in 300 cc. of water and add 6.97 grams of solid trisodium citrate dihydrate. Heat to boiling while stirring. The solution should be almost clear within ten minutes. Adjust pH to about 6 with 10 per cent sodium hydroxide (~13 cc.). Adjust volume to 400 cc. with boiled water and filter through quantitative paper into a sterile container.

The final concentrations in the above solution were  $\text{Na}^+ = \sim 0.26$  molar, zirconium =  $\sim 0.24$  M and about 0.24 M in citric acid. The composition of the final product would correspond to the formula  $\text{Na}^+[\text{ZrO}(\text{C}_6\text{H}_5\text{O}_7)_3]$ .

<sup>1</sup> The work described in this paper was completed in 1946 at the Argonne National Laboratory, Chicago, Illinois under Contract No. W-31-109-eng-3S.

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TABLE 1

*Acute toxicity of zirconium and thorium solutions after intraperitoneal injection at frequent intervals of large doses in rats*

COMPOUND*	CONCENTRATION, SOLUTION INJECTED	NUMBER OF INJECTIONS	TOTAL DOSE (AS METAL)	MORTALITY	LD <sub>50</sub> ± S.E.
Zirconyl ion dissolved in trisodium citrate	mgm./cc. as metal 24†	1	230	0/6	1,710 ± 80
		1	480	0/6	
		2	960	0/6	
		3	1,410	0/8	
		4	1,730	6/10	
		4	1,900	9/12	
		5	2,200	6/6	
Zirconyl ion dissolved in sodium gluconate	10.5‡	2	150	0/6	247 ± 40
		2	200	2/6	
		2	240	7/14	
		3	290	4/4	
		4	470	7/8	
		5	650	3/3	
Thorium nitrate dis- solved in trisodium citrate	5.0§	1	48.6	0/6	68 ± 12
		1	60.8	5/12	
		2	70	5/6	
		2	100	3/4	
		3	157	6/6	

\* Preparation given in text.

† Maximum single dose was 4 cc. Repeated injections were made at four hour intervals.

‡ Maximum single dose was 2.5 cc. Repeated injections were made at four hour inter-  
vals.

§ Maximum single dose was 2.5 cc.

TABLE 2

*Sub-acute toxicity of a zirconium solution\* after intraperitoneal injection in rats when administered in divided doses over an extended period*

AMOUNT IN EACH INJECTION†	NUMBER OF INJECTIONS PER DAY	NUMBER OF DAYS METAL GIVEN	TOTAL DOSE	MORTALITY
mgm./kgm.			mgm./kgm.	
245	1	8	1,960	1/12
490	1	4	1,960	0/4
490	2 (6 hrs. apart)	2	1,960	1/4
450	1	5	2,250	1
		7	3,150	1
		8	3,600	0
450	2 (6 hrs. apart)	3.5	3,150	2
		4	3,600	2

\* Essentially a solution of zirconyl ion in trisodium citrate. Details of preparation given in text.

† The solution contained 24 mgm. as zirconium per cc.

A satisfactory solution of zirconium in sodium gluconate is prepared as follows:

Eight hundred cc. of a solution containing 17.92 grams of sodium gluconate is brought to a boil and 30 cc. of zirconyl chloride solution containing 125-130 mgm. of zirconium per cc. is slowly added with constant stirring. The suspension is kept at the boiling point for fifteen minutes. After cooling, the suspension is centrifuged and the precipitate is washed with 200 cc. of water. It is then dissolved in 150 cc. of hot 0.2 M sodium gluconate and the solution is filtered into a sterile bottle.

The zirconium concentration in the sodium gluconate solution was about 20 mgm. per cc. The pH was 4.6.

The thorium containing solutions were prepared by adding 1.9 grams of trisodium citrate to a solution containing 1.8 grams of thorium nitrate and diluting to a final concentration containing 5 mgm. of thorium per cc.

**RESULTS AND COMMENT.** The data concerning the acute toxicity are given in table 1. The standard error was calculated by the simplified graphical method of Miller and Tainter (2).

Rats which received large frequent doses (table 1) of the zirconium or thorium compounds and survived thirty-six hours invariably recovered. The rats which died as a result of divided doses of the citrate compound of zirconium (table 2) lingered for periods as long as twelve days following their last injection.

The animals which received fatal doses of zirconium or thorium had nasal hemorrhages and considerable mucus in the respiratory tract. Upon autopsy considerable transudate was found in the peritoneal cavity. No gross damage to the liver or other organs was observed.

Following the administration of large single doses of zirconium or thorium compounds, the animals lost weight steadily for two to three days, after which time their weight eventually reached those of the controls. The larger the dose of these metals administered, the greater was the maximum weight loss and the slower the recovery. These results are summarized in table 3. These same general relationships hold for the animals which received divided doses (figure 1) of the citrate salt of zirconium. However, since the total dose given was larger, it was found that some of the animals suffered a maximum weight loss of 27 per cent before recovering.

Histologic examination of sections of the kidney, adrenal, spleen, lymph node and heart was made one month after the injection date on rats which had received 1730, 960 and 480 mgm./kgm., respectively, of zirconium as the citrate salt. No lesions were found. Hematologic examination was made on two rats which had received 50 mgm./kgm. of zirconium as the citrate salt by intravenous injection three days previously. The total and differential leucocyte counts, hemoglobin concentrations and clotting times did not show any significant differences from the same determinations on the blood of the uninjected control animals.

It has been reported previously (1) that 80-90 per cent of the zirconium (citrate salt) is excreted in the urine within the first twenty-four hours after intraperitoneal injection. Very little zirconium was excreted in the feces. The greater toxicity of the gluconate salt of zirconium as compared to the citrate salt may be due to a slower rate of excretion of the former.

The surviving rats appeared to be normal in all respects six months after receiving their last injection.

It is of interest to note the striking difference in toxicity between the sub-group IV elements, Ti, Zr, Hf and Th as compared to the sub-group V elements, V and Cb. The former, compound for compound, are relatively non-toxic (disregarding the long range radiation effects of Th) while the latter elements are decidedly toxic. This being the case, it seems reasonable to assume the remaining elements of Sub-group V elements, namely Ta and Pa, would be quite toxic if administered in the form of soluble salts. However, the relative toxicity of the citrate complexes of the group V elements is an unexplored problem.

Thorium nitrate when dissolved in sodium citrate appears to be less toxic

TABLE 3

*Weight changes in rats following the intraperitoneal injection at frequent intervals of large doses of zirconium and thorium solutions\**

COMPOUND	TOTAL DOSE (AS METAL)	MAXIMUM AVERAGE WEIGHT LOSS AFTER LAST INJECTION†	AVERAGE TIME ELAPSED BEFORE RETURN TO WEIGHT OF CONTROLS
Zirconium (citrate salt)	mgm./kgm.	%	days
	1,410	8	16
	960	6	8
	480	2	6
	230	1	2
Zirconium (gluconate salt)	242	13	20
	200	11	18
	150	10	10
Thorium (citrate salt)	60.8	8	10
	48.6	4	9
	23.7	5	9
	11.6	2	6

\* Solutions used and the conditions of injection are the same as described in table 1.

† In all cases the maximum weight loss took place two to three days after the last injection.

to dogs than it is to rats. It has been found that even when 250 mgm./kgm. of the citrate salt of thorium is administered intravenously to a dog no effect on circulation, or any other function, is observed (3). Undoubtedly a major reason for the relative nontoxicity of the citrate salts of both Th and Zr is the fact that they are chemically bound as soluble anions, thus inhibiting the protein precipitating action of these otherwise highly charged cations.

According to Fairhall (4) zirconium has only mild pharmacologic action and may even lack physiologic effects in small amounts. Lendle (5) cites work of Kaestle (6) who found that no toxic symptoms resulted from the intragluteal injection of several grams of the dioxide in rats after a period of eight months. Kaestle further showed that the injection of 0.4 gram/kgm. of soluble zirconium

salts to a rabbit produced no symptoms of intoxication. Daily feeding to a dog of 1 gram/10 kgm. of soluble zirconium salts did not result in any toxic symptoms after 146 days (5).

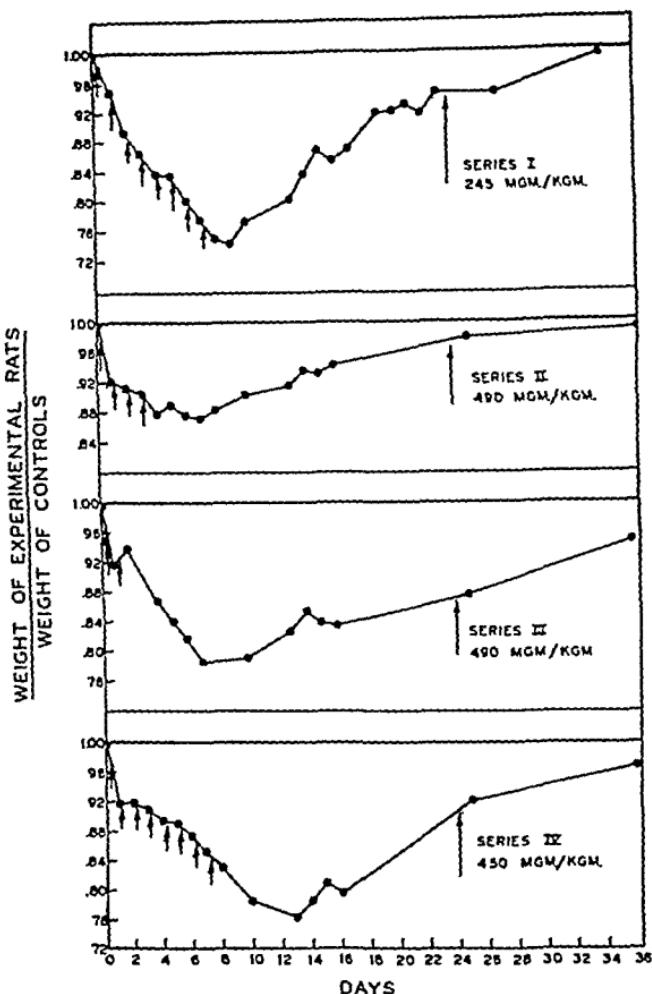


FIG. 1. WEIGHT CHANGES IN SURVIVING RATS FOLLOWING INTRAPERITONEAL INJECTIONS OF ZIRCONIUM CITRATE ADMINISTERED IN DIVIDED DOSES OVER AN EXTENDED PERIOD

The solutions used and the condition of injection are those described in table 2. Each arrow represents a separate dose. The concentration of the dose for each series is as given in the graph.

The remarkable innocuousness of the citrate salt of zirconium is encouraging in view of its promising effectiveness in the treatment of plutonium poisoning (1). Because of the probable radiation damage resulting from the use of thorium dioxide as a radio-opaque substance it would seem very worthwhile to investigate intensively the use of zirconium dioxide for this purpose. Kaestle (6) had used zirconium dioxide for x-ray visualization of the alimentary canal.

## SUMMARY

1. The acute toxicity in rats of the citrate and gluconate salts of zirconium and the citrate salt of thorium have been studied. The salts were administered by intraperitoneal injection at frequent intervals for not more than 20 hours in large doses and, in the case of zirconium citrate, in divided doses over a period of two to eight days.

2. The LD<sub>50</sub>'s and the standard errors for the experiments where the animals received large frequent doses of the above salts were determined.

3. Rats which survived 36 hours following the large frequent doses of the zirconium and thorium salts invariably recovered and exhibited no toxic symptoms 6 months later. No hematologic or histologic effects of zirconium (citrate) were found.

4. As much as 3,600 mgm./kgm. of zirconium (citrate) was tolerated by rats when administered by intraperitoneal injection once daily in doses of 450 mgm./kgm.

We are grateful to Miss Ruth P. Rhoades for carrying out the histological examinations and to Miss Rose Hunter and Mrs. Myrtle Karcher for their valuable services. We are indebted to members of the Biology Division and in particular to Dr. Hermann Lisco and Dr. Miriam Finkle for their advice on technical problems arising during the course of this work.

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# THE POTENTIATING EFFECT OF ANTIHISTAMINIC DRUGS UPON THE SEDATIVE ACTION OF BARBITURATES

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One of the drawbacks to the use of antihistaminic drugs for the alleviation of symptoms in allergy is the relative frequency with which undesirable side effects are encountered. The most common side reaction is drowsiness, which in some patients is of sufficient intensity to preclude the use of the drug during the day time.

There has been no test in animals for the sedative effect of these drugs, and without such a test, it has not been possible to estimate the possible sedative effect of new compounds on man. In doses considerably below the toxic level, the common antihistaminic drugs have no apparent effect upon normal animals. Loew, et al. (1) observed no evidence of drowsiness in dogs which had received diphenhydramine hydrochloride (Benadryl), 10 mgm./kgm. subcutaneously. In our laboratory, we have administered similar doses of pyranisamine maleate (Neo-Antergan) and other antihistaminic drugs to mice, rats, dogs, guinea pigs, and monkeys, without observing evidence of sedation. If the dose is increased to toxic levels, excitability and convulsions are seen. Mayer, et al. (2) observed this phenomenon in dogs given tripelennamine hydrochloride (Pyribenzamine) intravenously, and we have seen it in mice and rats as well as dogs, after toxic doses administered subcutaneously, intraperitoneally, and orally.

It is evident, then, that whatever potentially sedative effect antihistaminic drugs may have in animals is masked by a co-existing excitatory effect. In low doses, the two effects may cancel each other, and in high doses the excitatory effect predominates. It occurred to us that it might be possible to demonstrate a sedative action of these drugs in animals by administering a drug of known sedative action, and observing the effect of an antihistaminic drug when superimposed upon it.

**MATERIALS AND METHODS.** Rockland mice of both sexes, weighing 17 to 35 grams, and guinea pigs weighing about 500 grams, were used in these experiments. The antihistaminic compounds were injected subcutaneously, in aqueous solution. The barbiturate was administered intraperitoneally about one-half hour after the antihistaminic drug. All solutions were of such strength that 0.2 ml. of solution was used for each 20 grams of body weight.

The barbituric acid derivatives used were hexobarbital soluble (Evipal soluble, Winthrop) and pentobarbital sodium (Nembutal, Abbott). Antihistaminic compounds tested were *N'*-(*p*-methoxybenzyl)-*N'*-(2-pyridyl)-*N*,*N*-dimethyllethylenediamine (Neo-Antergan), *N'*-(2-pyridyl)-*N'*-benzyl-*N*,*N*-dimethyllethylenediamine (Pyribenzamine),  $\beta$ -dimethylaminoethyl benzohydryl ether (Benadryl), and *N*-(*β*-dimethylamino- $\alpha$ -methylethyl)-

phenothiazine (3277 R.P.).<sup>1</sup> Experiments were performed using the following combinations of drugs (in mice): antihistaminic 10 mgm./kgm., Nembutal 50 mgm./kgm.; antihistaminic 10 mgm./kgm., Evipal 60, 80, or 100 mgm./kgm.; antihistaminic 20 mgm./kgm., Evipal 40 mgm./kgm.

Most of the experiments were done in mice. The animals were placed in groups of 5 or 6. In practice, it was found possible for one person to observe 3 groups (15 to 18 animals) at once. Two groups received antihistaminic drugs (usually a different drug for each group) in addition to barbiturate, and the third group barbiturate only. The time of injection was noted, and thereafter observations were made of the animals' condition at regular intervals. For most of the experiments, observations were made at 5 minute intervals, beginning 15 minutes after the injection of barbiturate, and continuing until the last animal had awakened. When the per cent of animals awake was plotted, in probits<sup>2</sup>, against log of time after injection, a straight line was obtained. Therefore, the response to each treatment could be adequately characterized by the geometric mean waking time and its standard error.

The stages of awakening of mice from barbiturate-induced sleep have been described by Carmichael (4). However, for the purposes of this study, it was found to be most convenient to classify an animal as either "asleep" or "awake". The criteria for considering an animal awake were: ability to walk in normal fashion, eyes wide open, fur not erected, nose and vibrissae in characteristic motion, and efforts to escape when picked up. After a

TABLE 1

*The effect of antihistaminic drugs in prolonging the sedative action of Evipal in mice*  
(Figures in parentheses are numbers of animals)

GROUP	DRUG	EVIPAL 100 MG.M./KG.M.	EVIPAL 100 MG.M./KG.M. WITH ANTIHISTAMINIC 10 MG.M./KG.M.	DIFFERENCE
I	Pyribenzamine	36.7 ± 1.1 (47)	40.7 ± 1.2 (52)	4.0 ± 1.6, P = .015
II	Neo-Antergan	37.1 ± 1.1 (45)	42.3 ± 1.3 (49)	5.2 ± 1.7, P = .003
III	Benadryl	39.3 ± 1.4 (35)	56.4 ± 1.9 (39)	17.1 ± 2.3, P = <.001
IV	3277 R.P.	42.4 ± 1.0 (38)	56.5 ± 1.2 (43)	14.1 ± 1.5, P = <.001

In practice, the end point was not difficult to judge, and was quite sharp, except in the case of some animals receiving Benadryl and 3277 R.P., in which the awakening seemed more gradual.

RESULTS. The combination of Evipal 100 mgm./kgm. and antihistaminic 10 mgm./kgm. gave consistent and clear cut results. The only differences noted with the other combinations were shifts in the positions and slopes of the curves. There appeared to be no qualitative difference in the results obtained.

Table 1 and figure 1 present details of the results obtained with Evipal 100 mgm./kgm. intraperitoneally after various antihistaminic compounds 10 mgm./kgm. The smooth curves in figure 1 were drawn from pooled data of all the experiments, with time in minutes after Evipal on the abscissa, and per cent

<sup>1</sup> The author gratefully acknowledges generous supplies of material from the following: Benadryl, Parke, Davis and Co.; Pyribenzamine, Ciba Pharmaceutical Products, Inc.; 3277 R. P., Societe des Usines Chimiques Rhone-Poulenc, Paris. The Neo-Antergan used in this study was manufactured by Merck & Co., Inc.

<sup>2</sup> A probit is a "probability unit", which is equivalent to one standard deviation in response. For discussion, see Bliss (3).

of animals awake on the ordinate. These curves were obtained from the data in the following manner: the per cent of the animals awake, in probits (3), was plotted against log time after Evipal. From the equations of the straight lines so obtained, coordinate values were computed for use in plotting exactly the asymmetrical sigmoid curves.

There was considerable variation from day to day in the exact position and slope of the control curve, but when this occurred there was a corresponding shift in the experimental curves. Although the mice used varied considerably in age, and were of both sexes, the animals in any one experiment were of uniform size, age, and sex. The control values given in table 1 are the geometric means

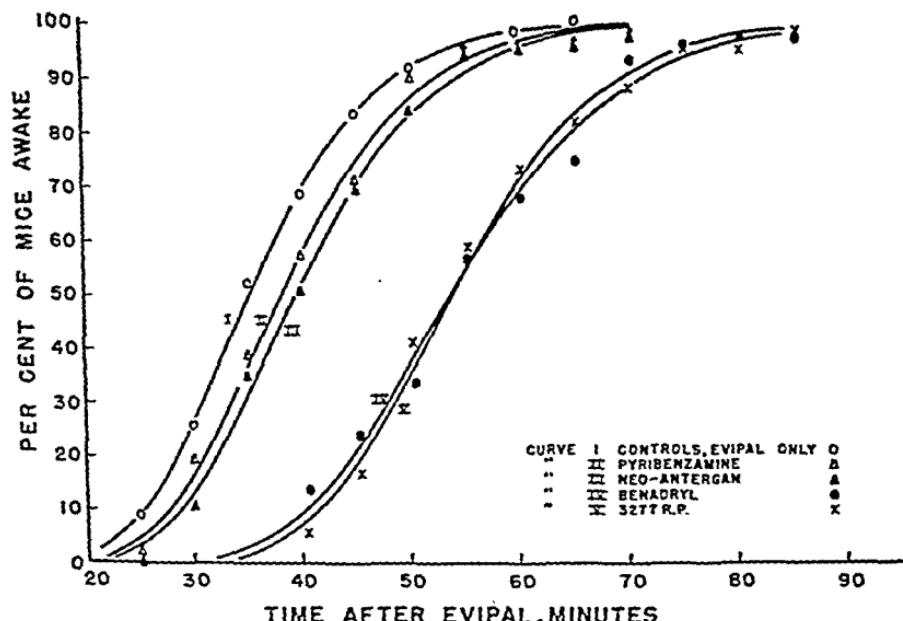


FIG. 1. THE EFFECT OF ANTIHISTAMINIC DRUGS, 10 MG./KGM., UPON THE WAKING RESPONSE CURVE OF MICE TREATED WITH EVIPAL 100 MG./KGM.

for the controls which were run concurrently with each drug as indicated, whereas the control curve in figure 1 is derived from pooled data from all the controls. The day to day variation made it necessary to run controls with each individual experiment.

These data show that Pyribenzamine and Neo-Antergan prolong the mean waking time by about 10 per cent, while Benadryl and 3277 R.P. prolong it about 40 per cent. In each instance, the difference between the control and the group receiving the antihistaminic drug was significant.<sup>2</sup> Thirty-five minutes after Evipal, nearly one-half the controls had awakened, while only about 30 per cent of the animals receiving Neo-Antergan or Pyribenzamine in addition to

<sup>2</sup>The author is indebted to Dr. W. H. Ott for statistical analysis of the data.

Evipal were awake. None of the animals receiving supplementary doses of Benadryl or 3277 R.P. had awakened within 35 minutes, although from the shapes of the curves, one might expect an occasional animal to awaken by this time when large numbers of animals are tested.

Table 2 shows the results obtained in similar experiments, using Nembutal 50 mgm./kgm. and Benadryl 10 mgm./kgm. It will be seen that the results were very similar to those obtained with Evipal 100 mgm./kgm. The mean waking time was 36 minutes for the controls receiving Nembutal only, and 54 minutes for the animals given Benadryl in addition.

One experiment was performed using guinea pigs instead of mice. Guinea pigs were found to be more susceptible than mice to the sedative effect of Evipal, 35 mgm./kgm. intraperitoneally sufficing to produce sleep. The mean waking

TABLE 2  
*Effect of Benadryl in prolonging the effect of Nembutal in mice*

TIME AFTER NEMBUTAL min.	BENADRYL 10 MG.M./KGM. + NEMBUTAL 50 MG.M./KGM. NUMBER OF ANIMALS		NEMBUTAL 50 MG.M./KGM. NUMBER OF ANIMALS	
	Asleep	Awake	Asleep	Awake
20	10	0	10	0
25	10	0	8	2
30	10	0	7	3
35	10	0	5	5
40	9	1	3	7
45	7	3	1	9
50	7	3	0	10
55	5	5	0	10
60	1	9	0	10
65	0	10	0	10
Mean waking time $\pm$ S.E.	$53.8 \pm 0.86$		$36.0 \pm 0.86$	

time of 6 guinea pigs receiving Evipal only was about 50 minutes, while 5 animals each receiving Benadryl 10 mgm./kkg. subcutaneously one-half hour before Evipal had an average waking time of 73 minutes. The end point of waking was not as sharp or as easy to judge as in mice.

DISCUSSION. The potentiating effect of Benadryl upon the sedative action of the barbiturates, according to this test, is much greater than that of Neo-Antergan or Pyribenzamine. Clinical reports also show that Benadryl produces a greater incidence of sedation in man than do Neo-Antergan or Pyribenzamine. With the use of Benadryl, McElin and Horton (5) reported side effects in 73 per cent of 74 cases, 44 per cent with drowsiness. Thacker (6) found side effects in 67 per cent of 72 patients, while Fuchs, et al. (7) observed drowsiness in 93 per cent of 30 patients. With Pyribenzamine, on the other hand, Arbesman, et al. (8) reported side reactions in 29 per cent of 495 patients. Bernstein, et al. (9)

compared the three drugs in 567 patients, and observed side effects in 23 per cent with Pyribenzamine and 27 per cent with Neo-Antergan. They did not report the incidence of side reactions with Benadryl, except to remark that it was higher than for the other two drugs. In all reports, sedation was the most frequent side reaction observed.

Clinical reports on the incidence of drowsiness or other side reactions in patients receiving 3277 R.P. are not available. On the basis of the results herein reported, we would expect 3277 R.P. to produce a rather high incidence of sedation.

#### SUMMARY

The antihistaminic drugs Pyribenzamine, Neo-Antergan, Benadryl, and 3277 R.P. prolong the sleep-producing effects of Evipal in mice. This potentiating effect was observed when an antihistaminic drug 10 or 20 mgm./kgm. was injected subcutaneously, followed one-half hour later by Evipal 40 to 100 mgm./kgm. intraperitoneally.

All the mice receiving Evipal 100 mgm./kgm. went to sleep, and, on the average, 50 per cent of the animals awakened in about 37 minutes. The mean waking time was prolonged about 10 per cent by Pyribenzamine and Neo-Antergan, and about 40 per cent by Benadryl and 3277 R.P.

Comparable results were obtained by a combination of Benadryl and Nembutal in mice, and Benadryl and Evipal in guinea pigs.

These results appear to correlate with the reported incidence of sedation as a side effect in patients receiving antihistaminic drugs.

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# STUDIES ON HYDROCARBON-EPINEPHRINE INDUCED VENTRICULAR FIBRILLATION

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In the years since Levy demonstrated that chloroform inhalation permits the initiation of ventricular fibrillation by stimulation of the sympathetic nervous system, or by amounts of injected epinephrine which are not ordinarily hazardous (1), a growing number of compounds has been found which share this property with chloroform. It now appears that if suitably examined, many unsubstituted or halogenated hydrocarbons, whether gaseous, liquid or solid, can be shown to possess this action. Compounds ranging in complexity of character from methane, cyclopropane and butane to xylene and 2,2 bis (p-chlorophenyl) 1,1,1 trichlorethane (DDT) have been demonstrated to predispose the ventricles to the fibrillating action of epinephrine (2).

On the other hand, epinephrine is not the sole sympathomimetic amine capable of producing ventricular fibrillation during the course of action of such hydrocarbons. Indeed, Meek has reported that several synthetic amines which contain the 1,2 dihydroxy benzene nucleus present in epinephrine are capable of initiating the gravest cardiac arrhythmias during cyclopropane anesthesia (3).

Thus, ventricular fibrillation occurring during anesthetization with chloroform, cyclopropane or ethyl chloride is not a capricious event but rather, an exhibition of a general pharmacological interaction between hydrocarbons and sympathomimetic amines. Although, during clinical anesthesia, as Levy pointed out initially, the sympathetic nervous system is the usual source of epinephrine (or sympathin), there is little evidence to suggest any essential difference between this and the effect of injected sympathomimetic amines. In order to obtain some further understanding of the mechanism by which the combined actions of these two groups of substances induce ventricular fibrillation the following experiments were performed.

**EXPERIMENTAL.** 1. *Comparative effect of hydrocarbons and non-hydrocarbons on the irritability of heart muscle.*

**Method.** The isolated papillary muscle preparation introduced by Cattell and Gold (4) was used with the muscle bathed in Locke's solution (5) containing sodium bicarbonate continuously buffered to pH 7.5 with 5 per cent carbon dioxide in 95 per cent oxygen in place of the usual phosphate buffer, and 0.01 M glucose. As reported by White and Salter (6) this resulted in preparations which showed little sign of failure for many hours. The muscle was stimulated by a thyratron oscillator discharge at a constant rate of approximately 60 per minute. A variable resistance was connected in series between the stimulator and the stimulating electrodes. Examination of the stimulus wave form on an electro-

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cardiograph revealed a straight line inverse relationship between the resistance and the stimulating voltage. No change in the shape of the stimulus was detected.

Following stimulation with a constant stimulus slightly above the threshold level during a 30 minute equilibration period, the stimulus was decreased by small decrements until the papillary muscle did not contract in response to any of 4 successive stimuli of a given intensity. The preceding stimuli were considered to be of threshold level. The stimulus was increased to the initial level and a second determination made 15 minutes later. If the two determinations differed by more than 2 per cent, and a longer equilibration period did not result in a constant level of irritability, the muscle was discarded. Random variations in resistance due to deflection of the muscle from the electrodes by gas bubbles were minimized by the use of Y-shaped electrodes with the muscle lying in the fork of the Y.

Stock dilutions of the substance under test were freshly prepared in Locke's solution on the day of the experiment as follows: benzene 50 mgm./cc., chloroform 200 mgm./100 cc., ethyl ether, ethyl alcohol and acetone 1000 mgm./100 cc. A suitable amount of stock solution was added to the Locke's solution surrounding the muscle to bring to the desired level the concentration of the substance being tested.

Two minutes after the compound under study was added to the muscle bath the intensity of the threshold stimulus was again determined. The concentration was then increased and the procedure repeated. By replacing the Locke's solution after washing the preparation it was possible to test both chloroform and ether on the same 10 papillary muscles. In 7, ether was tested first. Benzene, alcohol and acetone were each tested on 5 muscles.

*Results.* Increasing the concentration of chloroform and benzene, the two hydrocarbons used in this experiment, was found to produce a marked increase in the threshold of irritability of cardiac muscle. On the other hand, the three non-hydrocarbons, ether, alcohol and acetone were much less active, in that order. See figure 1 and table 1.

All of these substances depressed the contractility of cardiac muscle in proportion to the degree of decreased irritability. In no instance did a spontaneous rhythm develop after the addition of these compounds. On the contrary, where a spontaneous rhythm had been present the action of these substances was to abolish it.

*2. Relation of drugs affecting the sympathetic nervous system to the initiation of fibrillation.* Although the actions of epinephrine on the circulatory system are involved, it is possible to divide sharply the excitatory and inhibitory actions. Advantage was taken of this fact to perform the following experiments.

*Method.* Each cat was anesthetized with Dial-urethane (Ciba), 0.7 cc./kgm. of body weight administered intraperitoneally; the trachea was cannulated and the carotid artery blood pressure and lead II of the electrocardiogram were recorded. Either chlorazol fast pink or heparin was the anticoagulant. Petroleum ether, a mixture of the lower hydrocarbons of the aliphatic series, (b.p. 35-60°C.), was used because of its convenient physical properties.

The response of each cat to the intravenous injection of 30 micrograms per kilogram of the amine to be tested was established. Ventricular fibrillation never occurred. When the effects of the amine had completely receded, 0.1 cc./kgm. of petroleum ether was administered intratracheally and the response established. After an interval of at least 15 minutes, another 0.1 cc./kgm. of petroleum ether was administered intratracheally, followed in 30 seconds by an intravenous injection of 30 micrograms per kilogram of the amine under test. If necessary, fifteen or more minutes later the test was repeated. A third trial was

sometimes necessary in which 0.2 cc./kgm. of petroleum ether and 60 micrograms per kilogram of amine were used.

Epinephrine hydrochloride, nor-epinephrine hydrochloride (*Arterenol*) and N-isopropyl epinephrine hydrochloride (*Isuprel*) were used. Dilutions containing the desired dose in 2 cc. of physiological saline were prepared from refrigerated 1:10,000 stock solutions. Each injection was made in 20 seconds. When N,N-dibenzyl- $\beta$ -chloroethylamine hydrochloride (*Dibenamine*) was used it was dissolved in propyleneglycol and injected very slowly intravenously 7 to 20 minutes before the fibrillation test was performed.

The effect of concentration on the threshold stimulus

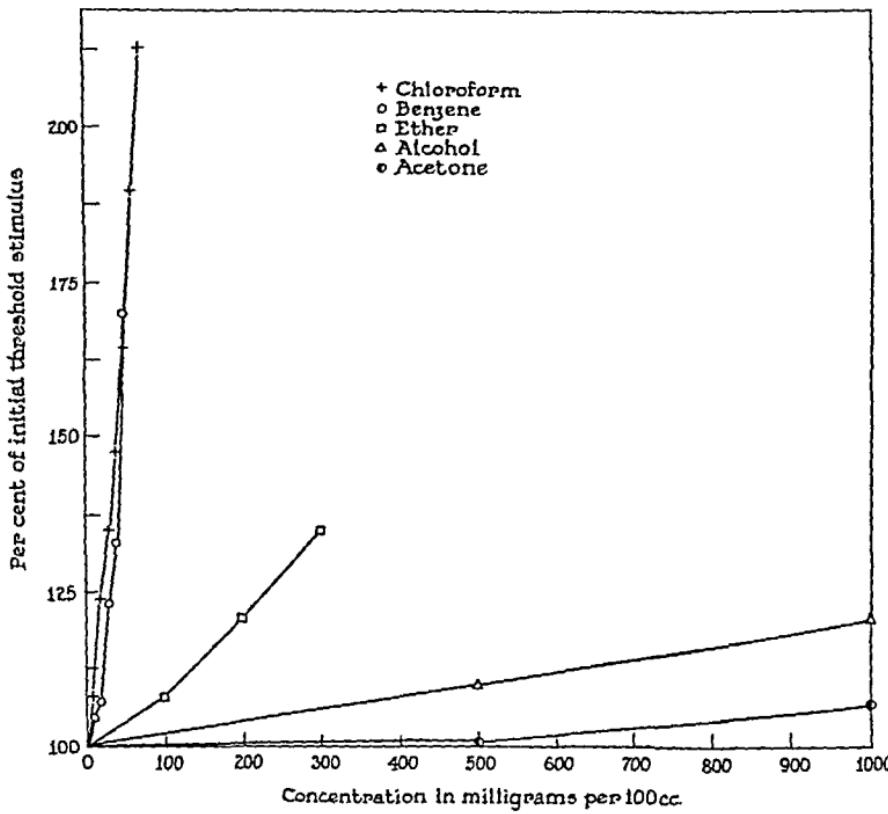


FIG. 1

**Results.** From the data of table 2 it is clear that the excitatory actions of epinephrine are responsible for the development of ventricular fibrillation. Nor-epinephrine, which exerts only excitatory actions, is probably equal to epinephrine in effectiveness; while N-isopropyl epinephrine, a purely inhibitory compound, is incapable of initiating ventricular fibrillation, although it did produce other irregularities.

Prior administration of 3 mgm./kgm. of Dibenamine to 10 cats did not alter the vasopressor effect of either 10 or 30 micrograms per kilogram of epinephrine. When epinephrine, 30 or 60 micrograms per kilogram, was injected into the same

10 cats during petroleum ether inhalation, all the cats developed heterotopic ventricular tachycardia and elevated blood pressure, but only 2 actually developed ventricular fibrillation.

*3. Electrocardiographic observations.* In the experiments described above, electrocardiograms were taken usually to confirm the presence or absence of ventricular fibrillation. The actual onset of the ventricular fibrillation was recorded in 11 instances. A characteristic pattern was noted in each of the records, consisting of a QRS complex smaller than most QRS complexes, followed by a T-wave of higher voltage than the QRS.

TABLE 1  
*The effect of concentration on the threshold of myocardial irritability*

MGM./100 CC.	CHLOROFORM	BENZENE	ETHER	ALCOHOL	ACETONE
10	108% (0.084 mM.)	104% (0.13 mM.)			
20	124% (0.17 mM.)	107% (0.26 mM.)			
30	135% (0.25 mM.)	123% (0.38 mM.)			
40	148% (0.34 mM.)	133% (0.51 mM.)			
50	164% (0.42 mM.)	170% (0.64 mM.)			
60	190% (0.50 mM.)				
70	213% (0.59 mM.)				
100			108% (1.4 mM.)		
200			121% (2.7 mM.)		
300			135% (4.1 mM.)		
500				110% (11 mM.)	101% (8.6 mM.)
1000				121% (22 mM.)	107% (17 mM.)

At the peak of the T-wave, a rapid deflection opposite in direction to the initial T-wave deflection occurred, accompanied by a sudden fall to zero of the carotid artery pressure. This deflection is considered the beginning of ventricular fibrillation. Two typical examples are presented in figure 2. The significance of the electrocardiographic pattern cannot be determined until further studies are made.

In none of the records was there any evidence of the acceleration described by Harris and Moe (7) as initiating fibrillation under certain circumstances.

**DISCUSSION.** The results of the experiments on myocardial irritability demon-

TABLE 2

Showing the frequency of ventricular fibrillation in cats following the administration of sympathomimetic amines during hydrocarbon inhalation in the presence and absence of adrenergic block:

AMINE HCl	NUMBER TESTED	NO FIBRILLATING AFTER 30 γ/KGM OF AMINE	NUMBER FIBRILLATING AFTER 60 γ/KGM OF AMINE BUT NOT AFTER 30 γ/KGM	TOTAL NUMBER FIBRILLATING	PER CENT FIBRILLATING
Epinephrine .. .	20	14	4	18	90
Epinephrine (after 3 mgm./kgm. Dibenamine)	10	1	1	2	20
Nor-epinephrine	5	2	3	5	100
N-isopropyl epinephrine	9*	0	0	0	0

\* In 4 of these the descending arch of the aorta was compressed about 10-15 seconds after the N-isopropyl epinephrine had been injected, raising the carotid pressure about 50 mm. of Hg.



FIG. 2. AN ENLARGEMENT OF TWO TYPICAL ELECTROCARDIOGRAMS OF CATS (LEAD II) SHOWING THE ONSET OF VENTRICULAR FIBRILLATION AFTER THE ADMINISTRATION OF PETROLEUM ETHER AND EPINEPHRINE

The numbers identify the last QRS (1) and T waves (2) before the onset of ventricular fibrillation (3). (Retouched)

strate that chloroform and benzene, two hydrocarbons, raise the threshold of irritability very greatly, while ether, alcohol and acetone have comparatively little effect. Changes in chloroform concentration of 5 mgm. per 100 cc. produced a change of 4 to 6 per cent in the threshold of myocardial irritability, while

a change of 5 mgm. per 100 cc. in ether concentration produced a change of only 0.3 to 0.5 per cent in the threshold. It may reasonably be assumed that all parts of the heart do not contain exactly the same concentration of a volatile anesthetic during inhalation anesthesia, particularly during induction and recovery. In addition to the usual variations in the response of a population (here myocardial cells) to a drug there may be some variation in concentration between different parts of the heart due to fluctuations in concentration of the gas in the inspired air, differences in rate of flow between small branches of the coronary arteries, etc. Therefore, it seems likely that during the inhalation of hydrocarbons, which markedly reduce myocardial irritability, some portions of the myocardium are less irritable than others, and therefore, small, temporary blocks are produced. There was no evidence that the hydrocarbons themselves induced ventricular automaticity, demonstrating the necessity for the concurrent presence of sympathin or sympathomimetic amines. In this respect it is evident that norepinephrine is fully as potent as epinephrine in producing ventricular fibrillation during hydrocarbon inhalation, while N-isopropyl epinephrine is not.

Although at present there is insufficient work to permit exact interpretation of the electrocardiographic findings, certain facts appear suggestive. The regular occurrence of a very low voltage QRS complex just preceding fibrillation, albeit recorded in a single lead (II), in a heart with decreased myocardial irritability permits the inference that fibrillation may have developed as the result of the stimulation and contraction of only the least depressed portions of the ventricle with subsequent re-entry in the manner described by Wiggers (8). Studies are planned which will explore this concept further.

The effectiveness of Dibenamine in preventing ventricular fibrillation from epinephrine and cyclopropane was well established by Nickerson and Goodman (9). However, the doses of Dibenamine used (20 mgm./kgm.) were such as to reverse completely the pressor effects of epinephrine. It is now apparent that Dibenamine is an efficient protecting agent, even in doses (3 mgm./kgm.) which do not reverse the pressor effects of epinephrine. The mechanism is obscure. Although ventricular fibrillation was prevented by these doses of Dibenamine in 80 per cent of the trials, heterotopic ventricular rhythms developed as frequently as in experiments without Dibenamine. This latter phenomenon was seen by Moe and his coworkers when dogs which had received Dibenamine (20 mgm./kgm.) were injected intravenously with epinephrine during cyclopropane anesthesia and the blood pressure was maintained by a pressure regulator or forced to rise by aortic compression (10). It is possible that the relative anti-fibrillatory effectiveness of Dibenamine in doses which do not reverse the pressor effects of epinephrine is related to the quinidine-like action of Dibenamine described by Acheson, Farah and French (11).

#### SUMMARY AND CONCLUSIONS

1. Chloroform and benzene raise the threshold of myocardial irritability very markedly, while ether, alcohol and acetone raise it but slightly.
2. Dibenamine exerts a protective action against hydrocarbon-epinephrine

induced ventricular fibrillation in doses which do not reverse the pressor effects of epinephrine.

3. Nor-epinephrine produces ventricular fibrillation during hydrocarbon inhalation, while N-isopropyl epinephrine does not.

4. A characteristic electrocardiographic pattern is seen just before the onset of ventricular fibrillation.

#### ACKNOWLEDGMENTS

The authors wish to thank Dr. William Gump of the Givaudan-Delawanna Company, who supplied the Dibenamine, and Dr. M. L. Tainter of the Sterling-Winthrop Research Institute who supplied the N-isopropyl-epinephrine (Isuprel), and through Dr. Benjamin Zweifach, the nor-epinephrine (d,l-Arterenol).

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# THE INHIBITION OF THE CHOLINESTERASE OF RAT BRAIN BY METHADON

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The peripheral and central effects of methadon are very similar to those of morphine. Scott et al. (1) have shown that the drug injected into dogs produces cardiac slowing, salivation, increased intestinal motility which is antagonized by atropine, constipation due to spasm after prolonged administration, and respiratory depression. Methadon, like morphine, raises the blood sugar of dogs (2), and this effect of both drugs decreases equally after a series of injections. In man (3), methadon in 15-30 mgm. doses slows respiration and the pulse rate and lowers systolic pressure. Like morphine it has little effect on the blood sugar level of man; upon intravenous administration it causes dizziness due possibly to a rapid lowering of blood pressure. The inhibition of cholinesterase by morphine (4, 5) may in part explain its cholinergic effects and it was of interest, therefore, to study the effects of methadon on this esterase. It may also be noted that meperidine (Demerol), which has already been shown to inhibit the esterase (6), increases intestinal motility and produces cardiac slowing in the dog (1). In man, however, it apparently has few cholinergic effects; in fact, its actions are more like those of atropine (7).

**EXPERIMENTAL.** Two rat brains were ground in a Waring blender with 50 cc. of distilled water. One drop of 2*N* acetic acid was added and the suspension was centrifuged. The supernatant was discarded, the solid was resuspended in water, acid was added and the suspension was re-centrifuged. This process was repeated five times in order to insure the removal of any blood esterase. The final material was suspended in 20 cc. of *M/20* phosphate buffer, pH 7.45, and 0.4 cc. of this was used in each Warburg vessel in a final volume of 3.2 cc. Phosphate-bicarbonate buffer was used and the hydrolysis measured in 95 per cent nitrogen-5 per cent carbon dioxide in the usual way.

Methadon (Lilly), containing 0.5 per cent chlorobutanol was made up in the buffer-bicarbonate mixture. This amount of chlorobutanol had no effect on the enzyme. For the sake of greater accuracy, freshly made up mecholyl bromide, rather than a salt of acetylcholine, was used as substrate. In order to determine the Michaelis constant it is necessary to use substrates the molar strength of which is not too far removed numerically from the value of the constant, otherwise the change of initial velocity with change in substrate concentration is too small. In the case of acetylcholine and true cholinesterase the constant is such that to satisfy this condition it would be necessary to use concentrations of acetylcholine so small as to make impossible accurate measurements of velocity. The value of the inhibitor-enzyme dissociation constant is the same whether

acetylcholine or mecholyl be employed as substrate. Only one isomer of mecholyl is hydrolyzed (8). Controls showed that the rate of hydrolysis in the absence of enzyme was negligible.

The evolution of carbon dioxide was followed at intervals of 10 to 15 minutes for two hours or more. During this period a large proportion or even all of the substrate was hydrolyzed; the rate therefore gradually fell off. It was found, however, that the course of the reaction could be accurately represented by the familiar equation:

$$\log \frac{a}{(a - x)} = ct$$

Results were fitted to this, and the velocity constant,  $c$ , was thus obtained. From this, the initial velocity, which is equal to  $c \cdot a$ , was calculated.

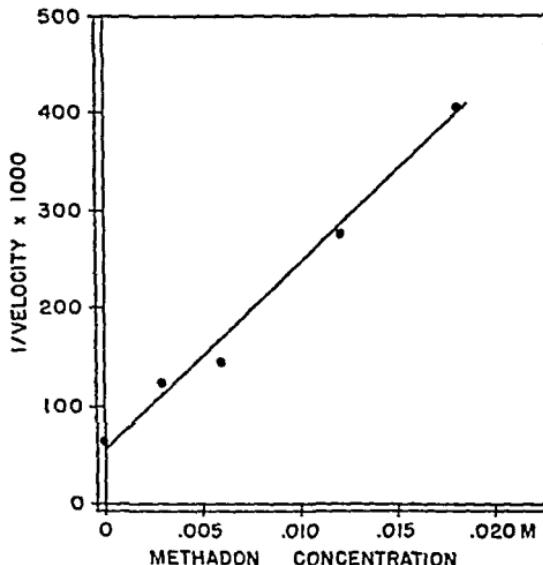


FIG. 1. THE EFFECT OF DIFFERENT CONCENTRATIONS OF METHADON ON THE RATE OF HYDROLYSIS OF MECHOLYL BY BRAIN CHOLINESTERASE

The Michaelis-Menten equation is usually given in the form:

$$v = \frac{Va}{a + K}$$

$v$ , being the initial velocity,  $V$ , the maximum velocity at "infinite" substrate concentration,  $a$ , substrate concentration and  $K$ , the dissociation constant. More useful are linear forms of this equation, and it has been previously pointed out (9) that it may be transformed into:

$$v = V - K \cdot c$$

where  $c$  is the velocity constant previously mentioned; it is, of course, equal to  $v/a$ .

Competitive inhibition is characterized by a change in slope from  $K$  to  $K(1 + i/K_i)$ ,  $i$  being the molar concentration of inhibitor, and  $K_i$  its enzyme

dissociation constant. In non-competitive inhibition the change is in the intercept,  $V$ . In an experiment using throughout the same enzyme preparation, values for the slope were found to be 0.000084 in the absence of methadon, and 0.0014 in the presence of this substance ( $0.00608 M$ ). Corresponding values for  $V$  were 0.000014 and 0.000012, respectively. The change is in the slope, and inhibition is competitive.

The effect of changing inhibitor concentration is best shown if the equation is rearranged with substitution of new constants,  $a$  and  $b$ , to give:

$$1/v = a + b \cdot i^n,$$

where  $n$  is the number of molecules of inhibitor combining with one molecule of enzyme. Data from an experiment are plotted in fig. 1. Since all points fall on a straight line it follows that the value for  $n$  must be 1.

It is now possible to calculate the inhibitor-enzyme dissociation constant. In two experiments without methadon the values of the mecholyl-enzyme dissociation constant,  $K$ , were  $6.5 \times 10^{-5}$  and  $8.4 \times 10^{-5}$ , the average being  $7.5 \times 10^{-5}$ . In two other experiments with a methadon concentration of  $0.00608 M$ , values for  $K(1 + i/K)$  were found to be  $1.1 \times 10^{-3}$  and  $1.4 \times 10^{-3}$ , respectively. Using an average value of  $1.2 \times 10^{-3}$ , the value for  $K$  is found to be  $4 \times 10^{-4}$ .

**DISCUSSION.** It probably is merely fortuitous that the three most powerful analgesics inhibit the cholinesterase *in vitro* and show evidence, although to different degrees, of cholinergic effects *in vivo*. Certainly many drugs which inhibit the enzyme have no analgesic effect. It is also true that some adrenergic compounds, such as amphetamine, are analgesics under certain conditions and these compounds may inhibit the amine oxidase of brain but they have no effect on the cholinesterase. Other analgesics such as salicylate and aminopyrine apparently have no effects on the autonomic system. Unless analgesia by different drugs is effected by different mechanisms, the inhibition of cholinesterase cannot be considered to be part of the analgesic action, but it does offer a probable explanation for the similar peripheral effects of morphine, methadon, and meperidine.

#### SUMMARY

Methadon inhibits the cholinesterase of rat brain. The characteristics of the inhibition are described.

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# THE ESTIMATION OF ACETANILIDE AND ITS METABOLIC PRODUCTS, ANILINE, N-ACETYL p-AMINOPHENOL AND p-AMINO-PHENOL (FREE AND TOTAL CONJUGATED) IN BIOLOGICAL FLUIDS AND TISSUES<sup>1</sup>

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A study of the fate of acetanilide in man required sensitive methods for the estimation of acetanilide and its metabolites in biological material. A method for aniline previously described (1) is not sensitive enough to estimate the small but significant amounts of this compound in plasma after the administration of acetanilide to man. Methods have been described for acetanilide, N-acetyl p-aminophenol and p-aminophenol (2). These are based on non-specific chemical reactions and are without rigorous appraisal as to whether the substances measured are identical with an authentic sample.

The determination of drugs in biological material has relied principally on spectrophotometric procedures. The question presents itself whether closely related compounds, derived in the body from the parent drug, are included in the measurement. Independent evidence must therefore be obtained as to whether the substance measured is identical with the known compound.

A technique previously described (3, 4) permits the identification and, to a considerable degree, the establishment of purity of the substance being measured. It involves a comparison of the distributions of the substance with those of the authentic substance in a two-phase system consisting of an organic solvent and water at various pH values. Dissimilar distributions indicate the presence of a substance different from the authentic compound. To escape detection, a transformation product must simulate the known compound to an improbable degree, in that it would have identical solubility characteristics in two solvents, and a similar dissociation constant. The procedure is suited to studies with biological material since it requires only a few micrograms of the particular substance. An additional advantage is that the information obtained on the solubility characteristics of the interfering substances may be applied towards a modification of the basic procedure to obtain the desired specificity.

Methods for the determination of acetanilide and its metabolic products in biological material are described below. The specificity of these methods has been appraised by the distribution technique.

**DETERMINATION OF ANILINE.** The aniline is isolated from the biological sample by extraction into benzene. It is returned to acid, diazotized and coupled with N(1-naphthyl) ethylene diamine at pH 7. The resulting dye is extracted

<sup>1</sup> The work described in this paper was supported by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

into a small volume of benzene, acidified and assayed in a spectrophotometer adapted to small volumes (5).

**Procedure.** Add 1 to 5 ml. of biological sample<sup>2</sup> at pH 6 or higher, (sample containing up to 2 micrograms of aniline) to 30 ml. of isoamyl alcohol-benzene<sup>3</sup> in a 60 ml. glass stoppered bottle. Shake for 10 minutes. Centrifuge the bottle. Transfer 20 ml. of the benzene phase to a 60 ml. glass stoppered bottle containing 6 ml. of 0.1N HCl. Shake for 5 minutes and centrifuge. Remove the organic phase by aspiration. Transfer 5 ml. of the acid phase to a 15 ml. conical glass stoppered tube. Add 0.5 ml. of 0.2 per cent sodium nitrite solution. Wait 10 minutes and add 0.5 ml. of 1 per cent ammonium sulfamate solution. After 3 minutes add 1 ml. of 50 per cent sodium acetate solution and 0.5 ml. of 0.2 per cent N(1-naphthyl) ethylene diamine dihydrochloride. Wait 20 minutes<sup>4</sup> and then add 0.5 ml. of 6N NaOH and 0.5 ml. of isoamyl alcohol-benzene. Shake for 1 minute thus extracting the dye into the benzene. Centrifuge and remove the aqueous layer by aspiration.

Transfer 0.3 ml. of the solvent to a micro colorimeter tube and add 0.05 ml. of a 25 per cent solution of trichloroacetic acid in ethylene dichloride. Mix by tapping the tube and determine the optical density of the solution at 570 mu using the Coleman Model 6 spectrophotometer adapted to micro spectrophotometry (5).

A reagent blank run through the procedure is used for the zero setting. This reagent blank should not read more than 0.020 when benzene plus trichloroacetic acid is used for the zero setting.

Standards are prepared by taking 5 ml. of standard aniline solution in 0.1N HCl, adding nitrite, sulfamate, acetate, coupling reagent and alkali, and extracting the dye into benzene as above. Five ml. of 0.1N HCl plus the other reagents are used for the zero setting. The optical densities were found to be proportional to concentration. An optical density of about 0.220 is obtained in the Coleman Model 6 spectrophotometer when 1 microgram of aniline is run through the procedure.

Aniline added to plasma and urine in amounts from 0.5 to 2 micrograms was recovered with satisfactory precision (98  $\pm$  5 per cent).

Acetanilide did not hydrolyze to aniline in stored samples of plasma and urine. However, acetanilide hydrolyzed at various rates in other tissues. Whole blood and homogenized organ tissues should therefore be acidified before storing when acetanilide is also present.

**DETERMINATION OF ACETANILIDE.** The acetanilide is isolated from the biological material by extraction into ethylene dichloride. The ethylene dichloride is evaporated and the residue taken up in acid. The acetanilide is hydrolyzed. The resulting aniline is diazotized and coupled with N (1-naphthyl) ethylene diamine at pH 7. The resulting dye is assayed in a spectrophotometer.

**Procedure for plasma and organ tissues.** Add 1 to 5 ml. of biological material<sup>2</sup> at pH 6 or higher, (sample containing up to 20 micrograms of acetanilide) to 30 ml. of the isoamyl

<sup>2</sup> Organ tissues and feces are prepared for analysis by emulsification in acid as described in a previous paper (4).

<sup>3</sup> Solvents are purified by successive washings with 1N NaOH, 1N HCl, and three washings with water. Reagent grade isoamyl alcohol (1.5 ml. per 100 ml. of solvent) which has been similarly treated is added to each solvent to minimize the absorption of the compound from the solvent onto the glass surface. The ether, reagent grade, should be washed the day it is used. Technical grades of benzene and ethylene dichloride are adequate.

<sup>4</sup> In the determination of larger amounts of aniline such as may be found in urine, the solution of the dye is acidified with 0.5 ml. of concentrated HCl and the optical density of the resulting solution determined directly at 550 mu.

alcohol-ethylene dichloride<sup>3</sup> in a 60 ml. glass stoppered bottle. Dilute the aqueous phase to 5 ml. if its volume is smaller than this. Shake for 10 minutes. Transfer the contents to a 50 ml. tube and centrifuge. Remove the aqueous phase by aspiration. Transfer 20 ml. of the solvent phase to a 50 ml. wide-mouthed, graduated conical tube and evaporate on a boiling water bath to about 0.25 ml. A stirring rod in the tube aids evaporation. Add about 8 ml. of 1N HCl and heat for one hour on a boiling water bath. Then add 1 drop of phenolphthalein indicator and neutralize with 6N NaOH. Adjust dropwise to the acid side with 1N HCl. Add 0.6 ml. of 1N HCl and adjust the volume with water to exactly 6 ml. Transfer 5 ml. of the solution to a colorimeter tube. Add 0.5 ml. of 0.2 per cent sodium nitrite solution. Wait 10 minutes and add 0.5 ml. of 1 per cent ammonium sulfamate solution. After 3 minutes add 1 ml. of 50 per cent sodium acetate solution<sup>5</sup> and 0.5 ml. of 0.2 per cent N-(1-naphthyl) ethylene diamine dihydrochloride. After 20 minutes add 0.5 ml. of concentrated HCl. Determine the optical density of the resulting dye with a spectrophotometer at 550 mu. A reagent blank run through the above procedure is used for the zero setting. This reagent blank should give a negligible reading when 0.1N HCl is used for the zero setting.

*Procedure for urine.* Carry out the analysis as described for plasma through the hydrolysis step. A brown pigment is formed in the case of urine from subjects receiving acetanilide. To separate the aniline from this interference adjust the pH of the solution to 7 or higher, extract into benzene and proceed as in the aniline procedure<sup>6</sup>.

The distribution of acetanilide in an ethylene dichloride-water system is such that with volumes of 30 and 5 ml. respectively, about 90 per cent of the acetanilide is in the organic phase. Standards are prepared by handling known amounts of acetanilide in the same manner as the unknown. The optical densities were found to be proportional to concentration. An optical density of about 0.300 is obtained in the Coleman Model 6 spectrophotometer when 10 micrograms of acetanilide are run through the plasma procedure.

Acetanilide added to plasma, urine and homogenized organ tissues in amounts from 4 to 15 micrograms was recovered with adequate precision ( $99 \pm 5$  per cent).

Repeated analyses of individual samples of refrigerator stored samples indicated that acetanilide was stable in plasma and urine but hydrolyzed to aniline at various rates in other tissues. To prevent this decomposition whole blood and homogenized organ tissues were acidified before storing.

**DETERMINATION OF N-ACETYL p-AMINOPHENOL.** N-acetyl p-aminophenol is extracted from the biological material into ether. The extraction is augmented by saturating the aqueous phase with sodium chloride. The N-acetyl p-aminophenol is returned to alkali and hydrolyzed in acid to p-aminophenol. The p-aminophenol is diazotized and coupled with alpha naphthol to form a dye which is assayed spectrophotometrically. This reaction for p-aminophenol is less sensitive than the indophenol one described later. However, use of the latter reaction involves neutralization of an excess of acid with the attendant danger of overrunning to the alkaline side and the consequent destruction of p-aminophenol.

*Procedure.* Add 1 to 5 ml. of biological sample<sup>2</sup> at pH 9 or lower, (sample containing up to 50 micrograms of N-acetyl p-aminophenol) to a 125 ml. glass stoppered bottle containing

<sup>5</sup> Occasionally a slight turbidity persists at this stage. This turbidity is corrected for by reading the optical density of the solution at this stage before the addition of the coupling reagent and subtracting the reading from the final optical density of the dye.

<sup>6</sup> Acetanilide values in urine are corrected for aniline also present.

2.5 grams of sodium chloride and 50 ml. of isoamyl alcohol-ether<sup>2</sup>. Dilute the aqueous phase to 5 ml. if its volume is smaller than this. Shake for 15 minutes and centrifuge the bottle. Transfer 40 ml. of the ether phase to a 60 ml. bottle containing 5 ml. of 0.1N NaOH. Shake for 5 minutes and centrifuge. Remove the organic phase by aspiration. Transfer 4 ml. of the alkali phase to a test tube containing 1 ml. of concentrated HCl. Cover the mouth of the tube with a glass marble to prevent evaporation and place in a boiling water bath for 30 minutes. Cool, and transfer 4 ml. of the solution to a colorimeter tube. Add 0.5 ml. of 0.2 per cent sodium nitrite solution. After 20 minutes add 0.5 ml. of 1 per cent ammonium sulfamate solution. After 3 minutes add 0.1 ml. of a 12 per cent solution of resublimed alpha naphthol in alcohol followed by 2 ml. of 5.5N NaOH. Cool immediately by immersion in cold water to prevent decomposition of the alpha naphthol. After 5 minutes determine the optical density of the resulting dye at 510 mu.

A reagent blank is run through the procedure and is used for the zero setting. This reagent blank should give a negligible reading when 0.1N HCl is used for the zero setting.

The distribution of N-acetyl p-aminophenol in an ether saturated saline system is such that with volumes of 50 and 5 ml. respectively, about 90 per cent of the N-acetyl p-aminophenol is in the organic phase. Standards are prepared by handling standard amounts of acetyl p-aminophenol in the same manner as the unknowns. Standards are run concurrently with the unknowns since there is a small daily variation in the optical densities. An optical density of about 0.110 is obtained on the Coleman Model 6 spectrophotometer when 10 micrograms of N-acetyl p-aminophenol are run through the procedure.

Recoveries of N-acetyl p-aminophenol added to plasma, urine and organ tissues in amounts of 10 to 50 micrograms were adequate ( $100 \pm 4$  per cent).

**DETERMINATION OF p-AMINOPHENOL.** Free p-aminophenol is separated from the biological material by extraction into ether. It is then returned to acid and coupled with phenol in the presence of sodium hypobromite to form an indophenol dye. This dye is assayed spectrophotometrically.

Total conjugated p-aminophenol consisting of the acetylated and the hydroxy conjugated derivatives is hydrolyzed to free p-aminophenol by heating the acidified biological material under pressure.

**Procedure.** Add 1 to 5 ml. of biological material<sup>2</sup> at pH 7 to 8 to 50 ml. of the isoamyl alcohol-ether<sup>2</sup>, in a 60 ml. glass stoppered bottle. Dilute the aqueous phase to 5 ml. if its volume is smaller than this. Shake for 10 minutes and centrifuge the bottle. Transfer 40 ml. of the ether phase to a 60 ml. glass-stoppered bottle containing 6 ml. of 0.01N HCl. Shake for 5 minutes and centrifuge. Remove the organic phase by aspiration. Transfer an aliquot of the acid phase containing up to 15 micrograms of p-aminophenol to a colorimeter tube and dilute to 5 ml. with 0.01N HCl. Add 1 ml. of freshly prepared 1 per cent phenol solution. Then add 1 ml. of freshly prepared sodium hypobromite solution. This solution is prepared by adding bromine water to 1N sodium carbonate until the solution is slightly yellow. After 20 minutes determine the optical density of the resulting dye with a spectrophotometer at 620 mu.

**Procedure for total conjugated p-aminophenol.** Add 1 to 5 ml. of biological material<sup>2</sup> to 1 ml. of concentrated HCl in a 15 ml. graduated conical tube. Dilute the aqueous phase to 6 ml. if the volume is smaller than this. Heat the solution in an autoclave at 15 pounds pressure for 1.5 hours. Adjust the volume to exactly 6 ml. Transfer a 5 ml. aliquot to a 125 ml. glass stoppered bottle containing 5 grams of K<sub>2</sub>HPO<sub>4</sub>. Mix thoroughly. Add 50 ml. of the isoamyl alcohol-ether and estimate the p-aminophenol as above.

A reagent blank run through the above procedure is used for the zero setting. The reagent blanks should give a negligible reading when 0.01N HCl is used for the zero setting.

The distribution of p-aminophenol in an ether-water system is such that with volumes of 50 and 5 ml. respectively, about 85 per cent of the p-aminophenol is in the organic phase.

Some of the p-aminophenol is lost during the manipulations. This loss is negligible when p-aminophenol in the order of 20 micrograms for plasma and 40 micrograms for urine are run through the procedure. There are appreciable losses for smaller amounts. The loss varies with the nature of the biological material. For this reason standards consisting of

TABLE I

*Distribution of aniline and apparent aniline between benzene and water at various pH values*

The apparent aniline was obtained by extraction with benzene of the urine of two subjects. One subject received acetanilide and the other, aniline. The apparent aniline was returned to dilute acid. Aliquots of this solution and of an authentic solution of aniline were adjusted to various pH values and shaken with 5 volumes of benzene. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

URINE A			URINE B		
pH	Authentic aniline (a)	Apparent aniline from urine (b)	pH	Authentic aniline (c)	Apparent aniline from urine (d)
3.2	0.39	0.38	2.7	0.30	0.20
4.3	0.81	0.83	3.7	0.80	0.70
5.5	0.98	0.93	4.9	0.99	0.96
6.0	0.98	0.93	5.9	0.99	0.98
7.0	0.98	0.98	7.0	0.98	0.97
9.0	0.97	0.95	7.9	1.03	0.99
11.0	0.93	0.95	10.0	1.05	1.01

Column (b) apparent aniline from the urine of a subject receiving acetanilide: column (d) apparent aniline from the urine of a subject receiving aniline.

TABLE II

*Distribution of acetanilide and apparent acetanilide between water and various ethylene dichloride-petroleum ether mixtures*

The apparent acetanilide was obtained by extraction with ethylene dichloride, of the plasma of a subject receiving the drug. The ethylene dichloride phase was evaporated to dryness and the residue dissolved in water. Aliquots of this solution and of an authentic acetanilide solution were adjusted to pH 7.0 and shaken with equal volumes of the various ethylene dichloride-petroleum ether mixtures. The fraction of the compounds extracted with the various solvent mixtures is expressed as the ratio of the amount of compound in the organic phase to total compound.

PER CENT PETROLEUM ETHER IN ETHYLENE DICHLORIDE	AUTHENTIC ACETANILIDE	APPARENT ACETANILIDE FROM PLASMA
0	0.89	0.90
20	0.89	0.86
40	0.83	0.86
60	0.71	0.73
80	0.48	0.47
100	0.11	0.11

known amounts of p-aminophenol added to biological material and extracted as described above are run concurrently with the unknowns. An optical density of about 0.520 is obtained in the Coleman Model 6 spectrophotometer when 20 micrograms of p-aminophenol are run through the procedure. Two micrograms of p-aminophenol can be detected.

SPECIFICITY OF METHODS. There was a negligible blank for each method in normal biological material. The extent of the interference of other known or unknown metabolic products of acetanilide was studied by the distribution technique (3, 4). The distributions of aniline, N-acetyl p-aminophenol and p-aminophenol (obtained after hydrolysis of conjugated forms) between an organic solvent and water at various pH values were compared with those of the apparent

TABLE III

*Distribution of N-acetyl p-aminophenol and apparent N-acetyl p-aminophenol between ether and water at various pH values*

The apparent N-acetyl p-aminophenol was obtained by extraction with ether of the NaCl saturated plasma, of a patient receiving acetanilide. The compound was then returned to dilute alkali. Aliquots of this solution and an authentic N-acetyl p-aminophenol solution were saturated with NaCl, adjusted to various pH values and shaken with 5 volumes of ether. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	AUTHENTIC N-ACETYL P-AMINOPHENOL	APPARENT N-ACETYL P-AMINOPHENOL FROM PLASMA
7	0.91	0.86
9	0.85	0.83
10	0.61	0.66
11	0.57	0.60
13 (0.1 N NaOH)	0.0	0.0

TABLE IV

*Distribution of p-aminophenol and apparent p-aminophenol between ether and water at various pH values*

The apparent p-aminophenol was obtained by extraction with ether of the hydrolyzed urine of a patient receiving acetanilide. It was then returned to dilute acid. Aliquots of this solution and of an authentic p-aminophenol solution were adjusted to various pH values and shaken with 8 volumes of ether. The fraction of the compound extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	AUTHENTIC P-AMINOPHENOL	APPARENT P-AMINOPHENOL FROM HYDROLYZED URINE
3.2	0.005	0.003
4.3	0.09	0.09
5.5	0.58	0.56
6.0	0.56	0.60
7.0	0.70	0.68

compounds extracted from biological fluid. Acetanilide is a relatively neutral substance with a negligible dissociation at various pH's. Distributions of the apparent acetanilide from biological fluid were compared, therefore, in a two phase system consisting of ethylene dichloride-petroleum ether solutions in various proportions, and water at a constant pH.

The results with each of the substances indicated that within experimental error, the distributions of the apparent compounds from biological material were

the same as those of the authentic compounds (Tables I-IV). It was concluded that the substances measured in biological fluids were identical with the authentic compounds.

#### SUMMARY

Methods are described for the determination of acetanilide and its metabolites. The procedure for acetanilide permits estimation of as little as 4 micrograms of the compound, that for aniline as little as 0.25 micrograms, for N-acetyl p-aminophenol and total conjugated p-aminophenol as little as 10 micrograms.

A comparison of the solubility characteristics of the authentic compounds and the apparent compounds extracted from the plasma and urine of subjects receiving acetanilide, indicates that the methods have a high degree of specificity.

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# THE FATE OF ACETANILIDE IN MAN<sup>1</sup>

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Therapeutic agents, in general, undergo chemical alteration in the body to form derivatives of the parent compound. Isolation and pharmacological assay of the various transformation products may yield valuable information in the search for better therapeutic agents, in studies on the mechanism of drug action and in the elucidation of normal metabolic processes.

The therapeutic effect of a drug could be limited by a chemical change which results in a less active or an inactive product. Knowledge of the nature of this metabolic product may suggest the introduction of substituents in the parent drug that would prevent the transformation and thereby enhance its activity. On the other hand, it is possible that the parent drug itself is inert but produces a therapeutic effect incidental to the formation of a highly active metabolic product. Knowledge of the structure of this derived product may suggest a synthesis of more effective derivatives. Again, the toxicity of the drug could reside in a derived product the formation of which, if its structure be known, may be blocked by an appropriate modification of the parent drug.

Fundamental studies on the mechanism of drug action attempt to correlate the effect of a drug on enzymatic activity with its pharmacological activity. The compound possessing the pharmacological action may be a product derived in the body from the parent drug. It is obvious in this case that the studies should be made not with the parent drug but with the active derived product.

It is unlikely that special enzymes exist in the body for the chemical transformation of each drug. Rather, it is likely that a drug undergoes chemical change by becoming involved in biochemical reactions which ordinarily handle normally occurring substances. The presence of a particular enzyme system within the animal is indicated by the transformation of the foreign compound. Knox used the transformation of quinine to 2-hydroxy quinine by various tissues as an indicator in the isolation in relatively pure form of the so-called quinine oxidase. He found this enzyme normally to be involved in the metabolism of nicotinamide (1).

The study of drugs from the above points of view has been considerably facilitated by two recent developments: 1. a scheme whereby simple and sensitive analytical methods for nitrogenous bases in biological material may readily be devised; included in this scheme is a technique for identifying the substance measured by means of its solubility characteristics (2, 3); 2. the technique of counter-current distribution which has been applied to the fractionation and isolation of substances from biological material (4).

<sup>1</sup> This work was supported by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

The aniline analgesics were selected for study because of their relatively simple chemical structure. The present investigation is concerned with the fate of acetanilide in the body. Studies on other drugs will follow.

A recent monograph on acetanilide presents the conflicting views of various investigators concerning the metabolism of acetanilide (5). It is generally believed, however, that acetanilide owes both its therapeutic and its toxic action to its conversion in the body to p-aminophenol which is then conjugated with sulfuric or glucuronic acid.

Recent work, reported since the publication of the above monograph, indicated that in the human, 70-90 per cent of administered acetanilide is excreted in the urine as conjugated p-aminophenol (6). Neither free p-aminophenol nor aniline was found. N-acetyl p-aminophenol and hydroxyl-conjugated N-acetyl p-aminophenol were found in the blood and plasma, but no free p-aminophenol (7). The compounds found were identified only on the basis of non-specific chemical methods without proof that a single substance was being measured identical with an authentic compound. The same studies also showed that the occurrence of methemoglobin after the administration of acetanilide was not due to the formation of p-aminophenol (8).

**METHODS AND MATERIAL.** Acetanilide and its metabolites were identified and estimated by the methods described in the previous paper (9).

Methemoglobin was determined by a slight modification of the method of Evelyn and Malloy (10). The subjects used for the experiments described below were normal subjects or patients with various chronic diseases without renal or liver involvement.

**EXPERIMENTAL.** A single lot of acetanilide was used for the entire study. This was a commercially available sample which was assayed for purity by the counter-current distribution technique using a procedure involving 25 plates (4). The results indicated that the acetanilide was at least 99.5 per cent pure.

*Absorption and excretion of acetanilide.* Information was obtained relative to the absorption from the gastrointestinal tract and the renal excretion of acetanilide, and the part that each of these processes plays in the physiological disposition of the drug after the oral dosage. Human subjects received 2 grams of the drug daily, in two doses, 6 hours apart, over a period of 6 days. Urine and stool collections were made during the last 72 hours of administration. The drug recovered in the stools in each instance amounted to about 0.1 per cent of the administered dose, indicating almost complete absorption from the gastrointestinal tract. Previous experiments had shown that the drug was not destroyed after incubation in stool suspensions for 24 hours at 37°C. The urinary excretion, in these as well as in single dose experiments, accounted for only 0.1-0.2 per cent of the administered acetanilide. Therefore, almost all the drug underwent metabolic alteration in the body.

*Plasma concentration-time curves.* The plasma concentrations of acetanilide and the per cent methemoglobin after the administration of single oral doses of 1 and 2 grams to man, in the post-absorptive state, are shown in table I. The results shown here are typical of 15 such experiments. As a rule, the absorption was rapid and maximal plasma levels were reached within 1 to 2 hours. The

plasma level fell rapidly, the drug being almost completely metabolized in about 7 hours. The methemoglobin followed a similar curve but with some time-lag.

*Acetanilide and its transformation products found in urine.* Information concerning the metabolic products of acetanilide was obtained in the urine of subjects given 1 gram orally. The urines were secured for the succeeding 24 hours and examined for the drug and its transformation products. The urinary excretion of acetanilide and its transformation products subsequent to this time, was negligible. About 80 per cent of the drug was accounted for as conjugated N-

TABLE I

*Plasma acetanilide and per cent methemoglobin following oral administration of one and two gram doses of acetanilide to man*

TIME	SUBJECT D				SUBJECT E			
	1 gram		2 grams		1 gram		2 grams	
	Acetanilide mgm./L.	Methemo- globin per cent						
hrs.								
0	—	1.4	—	0.3	—	1.5	—	2.2
1	5.6	3.9	17.0	7.2	9.0	4.5	11.0	5.0
2	5.4	6.0	12.0	12.0	7.2	7.7	19.0	10.0
4	2.8	6.6	6.8	13.2	5.0	8.0	11.5	17.5
7	0.5	2.8	1.6	6.0	1.1	4.3	4.2	13.5

TABLE II

*The metabolic fate of acetanilide in man*

Recovery of acetanilide and its metabolic products from the urine of subjects given single oral doses of acetanilide.

The urine was collected over a period of 24 hours. The proportion of the various metabolites is expressed in percentage of the amount of acetanilide administered.

ACETANILIDE ADMINISTERED	ACETANILIDE	N-ACETYL P-AMINOPHENOL	CONJUGATED N-ACETYL P-AMINOPHENOL	ANILINE
grams	per cent	per cent	per cent	per cent
1.0	0.11	3.9	82	0.05
1.0	0.19	3.8	70	0.05
2.0	0.10	3.4	78	0.05
2.0	0.10	3.2	86	0.03

acetyl p-aminophenol,<sup>2</sup> about 4 per cent as free N-acetyl p-aminophenol, and traces as aniline. Less than 0.2 per cent of the parent drug was excreted unchanged. The results shown in table II are representative of 10 similar experiments. That the conjugated p-aminophenol also was acetylated was surmised from the absence of free amino groups in plasma or urine other than that due to the small amount of aniline present. The conjugated N-acetyl p-aminophenol

<sup>2</sup> The conjugated N-acetyl p-aminophenol is the total conjugated p-aminophenol less the N-acetyl p-aminophenol.

has been shown to be esterified at the OH group, partly with sulfuric and partly with glucuronic acid (7). Free p-aminophenol was not detected. An attempt was made to demonstrate the presence of a substance in urine or plasma capable of converting hemoglobin to methemoglobin. Urine or plasma from subjects receiving acetanilide was added to hemolyzed red cells. One hour incubation of this solution at 37°C. failed to result in the formation of methemoglobin.

It is to be noted that the above substances were not analyzed merely on the basis of non-specific chemical reactions but by methods which identified the material measured as a single substance identical with the authentic compound (9).

Additional evidence that the apparent p-aminophenol resulting from the hydrolysis of its conjugated derivatives in urine was a single substance rather than a mixture, and the actual isolation and precise identification of this substance was gained with the aid of Craig's counter-current distribution technique. The urine was acidified by the addition of  $\frac{1}{5}$  volume of concentrated HCl and heated in an autoclave at 15 pounds for 1.5 hours. The urine was now adjusted to pH 7 by the addition of K<sub>2</sub>HPO<sub>4</sub> and the apparent p-aminophenol extracted with ether. The apparent p-aminophenol was then returned to 0.1 N HCl. The material was then subjected to a counter-current distribution involving 8 transfers, according to the method of Craig (4). The distribution was effected in a series of separatory funnels, between the immiscible solvent pair: isoamyl alcohol and pH 5.8 phosphate buffer. The partition coefficients of the apparent p-aminophenol in each separatory funnel was then determined by measuring the concentration in each phase. The partition coefficients were found to be the same, within analytical error, for the apparent p-aminophenol in each separatory funnel. It was concluded, therefore, that the material which reacted chemically as p-aminophenol was not a mixture but a single substance.

The aqueous phases of the middle three separatory funnels were acidified with HCl. The total apparent p-aminophenol in each separatory funnel was transferred to the aqueous phases by shaking. The three aqueous phases were combined and the pH adjusted to 7. The apparent p-aminophenol was extracted into ether and then returned to dilute HCl. This latter phase was evaporated to dryness on a water bath. The residue was taken up in 30 per cent acetic acid, and benzaldehyde added to form a benzylidene derivative. After two recrystallizations from 30 per cent ethyl alcohol, crystals melting at 179–183°C. were obtained. The mixed melting point with the benzylidene derivative of pure p-aminophenol was unchanged. This showed that the apparent p-aminophenol isolated from urine was identical with the known substance.

*Acetanilide and its transformation products found in plasma.* The plasma levels of acetanilide and its transformation products after the oral administration of 1 gram doses of the drug are shown in table III. These results are representative of 13 similar experiments. It is seen that the acetanilide levels declined rapidly while those of N-acetyl p-aminophenol increased, suggesting that acetanilide was quickly oxidized to the latter compound. The plasma levels of acetanilide and N-acetyl p-aminophenol permitted an estimate of the amounts of these substances in the body. The calculation was based on the experimental finding for

dogs that both compounds are distributed fairly uniformly throughout total body water. Thus in the case of subject D, if the acetanilide were distributed in 70 per cent of the body weight, the amount of administered acetanilide which remained in the subject would correspond to 28 per cent in 2 hours and 2.5 per cent in 7 hours. The corresponding amounts for N-acetyl p-aminophenol in terms of the administered acetanilide would be 41 per cent in 2 hours and 11 per cent in 7 hours. A rise in conjugated N-acetyl p-aminophenol was coincident

TABLE III

*Plasma acetanilide, N-acetyl-p-aminophenol, conjugated N-acetyl-p-aminophenol and aniline after the oral administration of 1 gram of acetanilide to man*

TIME	SUBJECT D				SUBJECT C			
	Acetanilide	N-Acetyl p-Amino- phenol	Conj. N-Acetyl p-Amino- phenol	Aniline	Acetanilide	N-Acetyl p-Amino- phenol	Conj. N-Acetyl p-Amino- phenol	Aniline
hrs.	mgm./L.	mgm./L.	mgm./L.	mgm./L.	mgm./L.	mgm./L.	mgm./L.	mgm./L.
1	9.3	7.9	2.1	0.05	14.0	7.6	6.9	0.09
2	5.8	9.4	5.6	0.04	9.3	7.9	9.7	0.08
4	2.7	6.2	10.4	0.02	5.5	6.6	12.8	0.05
7	0.5	2.6	7.0	0.01	1.2	3.0	11.5	0.02

TABLE IV

*Distribution of acetanilide and aniline in the dog*

The distribution of acetanilide and aniline was examined in dog tissues. The studies were made 4 hours after the oral administration of 2.9 grams of acetanilide. The dog weighed 23 kilograms.

TISSUE	CONCENTRATION OF ACETANILIDE		CONCENTRATION OF ANILINE
	mgm./kgm.	mgm./kgm.	
Plasma.....	70		11.4
Whole blood.....	63		15.0
Kidney.....	59		11.2
Heart.....	60		10.3
Muscle.....	57		11.2
Lung.....	58		12.1
Liver.....	73		15.2
Cerebrospinal fluid.....	58		7.3
Brain.....	60		5.8

with a fall in free N-acetyl p-aminophenol as the latter compound is conjugated. Aniline levels were low in comparison with the other substances, suggesting that only a minor fraction of the acetanilide was deacetylated. However, as will be shown later, this small amount of aniline plays a significant role in the toxicity of acetanilide. No free p-aminophenol was demonstrated.

*Acetanilide and its transformation products found in tissues.* The distribution of acetanilide and aniline 4 hours after the oral administration of acetanilide to a dog, is shown in table IV. The concentration of acetanilide in most organ

tissues was about 80 per cent of that in plasma. The extent to which acetanilide was bound to the non-diffusible constituents of plasma, presumably plasma albumen, was determined by dialysis against isotonic phosphate buffer of pH 7.4 and at 37°C. for 18 hours. Visking membranes were utilized for the dialysis bags. None of the acetanilide was found to be bound in plasma. These results indicated that acetanilide was distributed uniformly throughout most body fluids with negligible localization in cellular tissues. Even in the cerebrospinal fluid and the brain the levels were such as to indicate little if any hindrance by the blood-brain barrier to the passage of the drug. In the above respects the acetanilide behaved like urea.

Aniline levels in various tissues were fairly uniform, although the cerebrospinal fluid and brain levels were distinctly lower than those in the other tissues. Here again, there was little if any localization of the compound in body tissues. Free p-aminophenol was not demonstrated in any tissue.

*Site and rate of the transformation of acetanilide in the body.* The role of the kidney and the liver in the transformation of acetanilide in the body was studied. The rate of the disappearance of acetanilide from the blood was compared with that of bromsulfalein, a compound presumed to be removed chiefly by the liver (11). Acetanilide and bromsulfalein were administered simultaneously to normal individuals in 3 experiments by means of a constant intravenous infusion at such a rate that the concentration of the substances in the peripheral blood remained unchanged. Under these conditions, the rate of the transformation of acetanilide by the body may be assumed to equal the infusion rate since urinary excretion of acetanilide is negligible. The concentration of acetanilide in the peripheral blood was compared with that in the blood leaving the kidney and the liver. The blood draining the organs was obtained by the venous catheterization technique (11).

The concentration of acetanilide in the renal vein blood did not differ significantly from that in the peripheral venous blood indicating that little, if any, role was played by the kidney in the transformation of acetanilide *in vivo*. On the other hand, the concentration of acetanilide in the hepatic vein blood was found to be considerably lower than that in the peripheral venous blood, indicating that considerable amounts of acetanilide had been transformed by the liver. If it is assumed that the drug was removed only by the liver, then the hepatic

blood flow per minute would be  $\frac{L}{X_1 - X_2}$  where L is the removal rate per minute

of the compound by the liver (equal to the infusion rate),  $X_1$  is the peripheral blood concentration and  $X_2$  is the concentration in the blood leaving the organ. The hepatic blood flow estimated from the data for bromsulfalein and acetanilide were in good agreement. This is in accord with the belief that the removal of acetanilide is also limited for the most part to the liver. The details of this work will be published subsequently.<sup>3</sup>

*Fate of aniline and N-acetyl p-aminophenol in the body.* The important role of aniline and N-acetyl p-aminophenol in the overall pharmacological action of acetanilide prompted a study of their fate in the organism. One hundred mgm.

<sup>3</sup> Berliner, R. W., and Kennedy, T. W.: To be published.

of aniline hydrochloride were administered orally to 6 human subjects. Urine was collected for a period of 24 hours. About 80 per cent of the aniline was found in the urine as conjugated N-acetyl p-aminophenol. The absence of free amino groups other than that which could be accounted for as aniline was taken as an indication that the conjugated p-aminophenol was also acetylated. No free p-aminophenol was demonstrated. Only about 0.6 per cent of the administered aniline was excreted unchanged. It is of interest that in the dog, the conjugated p-aminophenol resulting from the administration of aniline contained a free amino group. This fits the well-known observation that the dog does not acetylate amino groups.

One gram doses of N-acetyl p-aminophenol were administered to 6 human subjects. About 85 per cent of the administered compound was found in the urine as total conjugated p-aminophenol. No free aniline was demonstrated in plasma or urine. About 3 per cent of the compound was recovered unchanged. The remainder could not be accounted for.

*The role of aniline in the formation of methemoglobin.* Both methemoglobin and aniline were found in the blood of man after the administration of acetanilide.

TABLE V

*Correlation of aniline and methemoglobin levels in the blood after the administration of aniline and acetanilide to man*

SUBSTANCE ADMINISTERED	AMOUNT ADMINISTERED	PLASMA ANILINE MAXIMUM LEVEL		MAXIMUM METHEMOGLOBIN per cent
		grams	mgm./L.	
Acetanilide.....	2.0		0.07	13.5
Acetanilide.....	2.0		0.05	9.0
Aniline hydrochloride.....	0.10		0.07	11.5
Aniline hydrochloride.....	0.10		0.05	10.0

Methemoglobin was also found after the oral administration of aniline. This suggested that the methemoglobin in the blood after the ingestion of acetanilide might have been formed as a result of the aniline present. The evidence for this was that the amount of methemoglobin is correlated with the plasma aniline concentration subsequent to the administration of either acetanilide or aniline (table V). A similar relationship between the aniline and methemoglobin levels held also in the case of the dog. About 20 times as much acetanilide as aniline was required to produce the same amounts of methemoglobin in man. This indicated that only a small fraction of the acetanilide was deacetylated to form aniline. Whole blood, incubated for 1 hour at 37°C. with either drug at a concentration of 100 micrograms per ml. of blood showed no accumulation of methemoglobin. It is concluded from this that the hemoglobin in the body was not oxidized to methemoglobin directly by aniline, but by some product derived from it in the organism.

The nature of the actual methemoglobin-forming agent is not known. It has been considered to be p-aminophenol. This hypothesis is made unlikely since free p-aminophenol was not demonstrated in the blood after the administration

of either acetanilide or aniline. In addition, both *in vivo* and *in vitro* experiments have shown that considerable concentrations of free p-aminophenol in blood were required to promote the formation of a measurable amount of methemoglobin (8). It is possible that phenylhydroxylamine is the actual methemoglobin forming agent. One mgm. per kilo of this compound administered intravenously to a dog resulted in the conversion of 45 per cent of its hemoglobin to methemoglobin. This is a considerably greater quantity of methemoglobin than would be expected on the basis of a stoichiometric reaction between phenylhydroxylamine and hemoglobin. This suggests that phenylhydroxylamine is involved in a cyclic reaction in which the oxidizing compound is being reformed. Concerning the fate of phenylhydroxylamine in the body, a considerable fraction of the administered compound rearranges in the organism to p-aminophenol which is then excreted in the conjugated form.

TABLE VI

*A comparison of plasma N-acetyl p-aminophenol levels after oral administration of equimolecular doses of N-acetyl p-aminophenol and acetanilide*

The dose of N-acetyl p-aminophenol was 1 gram, and that of acetanilide was 0.9 gram.

TIME	PLASMA N-ACETYL P-AMINOPHENOL, MG.M./L.			
	Subject X		Subject Y	
	After N-Acetyl p-aminophenol	After Acetanilide	After N-Acetyl p-aminophenol	After Acetanilide
<i>hours</i>				
1	2.9	1.1	5.8	3.5
2	8.2	3.1	10.9	4.1
3	4.6	6.5	7.5	4.8
5	2.6	3.0	2.9	3.9
8	0	1.0	0	2.7

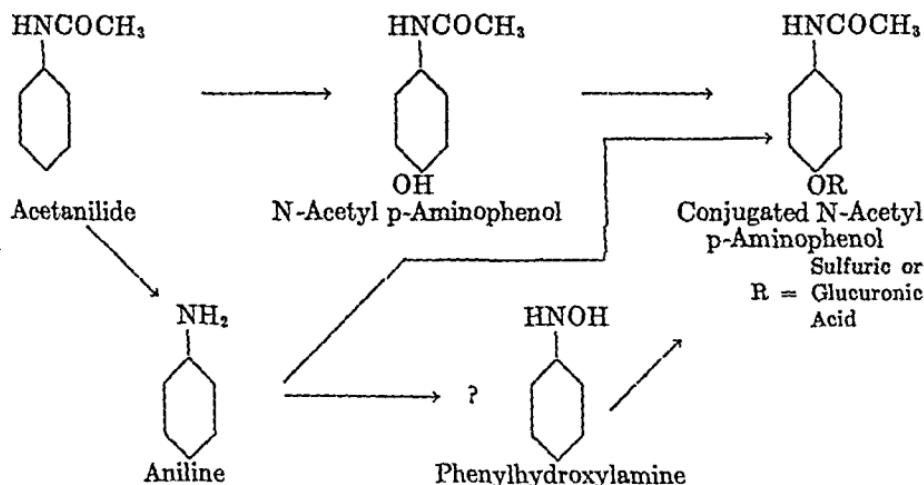
The characteristic toxic symptoms of acetanilide overdosage may be largely explained in terms of the anemic anoxia resulting from the formation of methemoglobin, and anemia due to the destruction of red cells (12). Aniline poisoning also results in methemoglobin formation and the destruction of red cells (12). It is probable that the overall toxicity of acetanilide is derived mainly from the small amount of aniline produced in the body.

*The role of N-acetyl p-aminophenol in the analgesic action of acetanilide.* N-acetyl p-aminophenol, which is found in both plasma and urine after the administration of acetanilide, appears to be the first step in the major route of the metabolism of the drug. The high concentration of this metabolite in the plasma prompted an appraisal of its analgesic effect. Studies conducted on human subjects, utilizing the Wolff-Hardy technique, showed N-acetyl p-aminophenol to be, dose for dose, about equal in analgesic activity to acetanilide (13). Plasma levels of N-acetyl p-aminophenol achieved after the administration of equimolecular doses of acetanilide and N-acetyl p-aminophenol were of the same order of magnitude, but those obtained after acetanilide persisted longer (table VI).

However, the peak levels of N-acetyl p-aminophenol, when this drug itself was given, were higher and were reached more rapidly, as is to be expected. The results are compatible with the assumption that acetanilide exerted its action mainly through N-acetyl p-aminophenol. Further studies are planned to ascertain whether acetanilide has any analgesic activity *per se* or whether its activity is predicated solely upon its conversion to N-acetyl p-aminophenol. The latter compound, administered orally, was not attended by the formation of methemoglobin nor, at least *in vitro*, did it destroy red cells. It is possible, therefore, that it may have distinct advantages over acetanilide as an analgesic, and it may well serve as a starting point for the synthesis of more effective agents.

Preliminary work in this laboratory has shown that acetophenetidin (p-ethoxyacetanilide), a well-known analgesic, is also transformed in the body to N-acetyl p-aminophenol.

**DISCUSSION.** The following scheme for the route of metabolism of acetanilide in the human is suggested by the observations described previously.



The main route of metabolism appears to involve two serial steps. The first of these is oxidation with the replacement of the hydrogen atom in the para position of the benzene nucleus by a hydroxy group to form N-acetyl p-aminophenol, an active analgesic. The second step is conjugation of this compound at the hydroxyl group with sulfuric or glucuronic acid. A minor channel of metabolism also involves several serial steps. Part of the acetanilide deacetylates to yield aniline. This compound then adds oxygen to the benzene nucleus to form p-aminophenol which is rapidly conjugated at both the amino and the hydroxy groups. Aniline is also the precursor of the substance, probably phenylhydroxylamine which is responsible for the formation of methemoglobin.

The route of metabolism of acetanilide in the organism differs from that expected on the basis of studies with animal tissues *in vitro*. It has been demonstrated that acetanilide is rapidly hydrolyzed to aniline when it is incubated with rat liver and kidney tissue (14). Yet the intact organism hydrolyzes only a

minor fraction of the drug to aniline, while the major fraction is oxidized to N-acetyl p-aminophenol. Results (unpublished) with other compounds make it apparent that studies *in vitro* concerning the fate of a drug may describe a pathway of metabolism which is of minor importance only, in the intact organism. Many factors come into play in the intact animal which may be eliminated in the simpler experimental conditions *in vitro*. This is not meant to imply that work with isolated tissues is not important. Such work can do much to clarify reactions which first have been shown to occur significantly in the whole animal.

#### SUMMARY

The route of metabolism of acetanilide in man was shown to be as follows: a minor fraction of the drug deacetylates to form aniline; this compound was shown to be the precursor of the substance which oxidizes hemoglobin to methemoglobin; the major fraction of the drug is oxidized to N-acetyl p-aminophenol; this compound is excreted in a conjugated form. The analgesic action of acetanilide is exerted mainly through N-acetyl p-aminophenol which is an active analgesic. The oxidation of acetanilide occurs mainly in the liver.

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# COMPARISON OF OUABAIN WITH STROPHANTHIDIN-3-ACETATE BY INTRAVENOUS INJECTION IN MAN

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In a previous study (1) on the behavior of several synthetic esters of strophanthidin in man, it was observed that these materials, after intravenous injection, developed their full action very rapidly, within 10 to 30 minutes, and that their persistence of action was very brief, the effects wearing off within a few hours. Since they were found to exert a typical digitalis action, the rapid onset and disappearance of their action provided a combination of characteristics with useful therapeutic possibilities. Accordingly, experiments were carried out to compare the curve of action of acetyl-strophanthidin (strophanthidin-3-acetate) with crystalline strophanthin or ouabain, the material usually considered for obtaining rapid digitalis action in man. The results of these experiments form the subject of this report.

The structural formulas of the two compounds are shown in figure 1. The ester has an acetyl group and the glycoside has the sugar rhamnose at carbon 3. There are also differences in the substitutions at carbon atoms 1, 10, and 11.

**METHOD.** The experiments were performed on patients with auricular fibrillation and varying degrees of heart failure. Table 1 summarizes the characteristics of the 7 subjects. They were selected from a group of 1500 patients in active attendance at our cardiac clinics, on the basis of the facts that they were without digitalis for several weeks, that they developed a rapid ventricular rate, and that they were subject to a degree of failure well within the limits of safety. The method was substantially similar to that used in previous studies (2). The patient was put to bed in the hospital. The ventricular rate was counted at the apex for one minute, several times daily, under conditions of quiet and rest. This was done during a preliminary control period of at least a week in order to establish the level of the resting rate without drug. A dose of the drug was administered and similar counts were made at intervals of several minutes until the maximum effect was in evidence, and then at less frequent intervals until the effect disappeared. Within a few days after the apex rate had returned to the control level, the patient was ready for another experiment which was carried out in the same manner. Simultaneous observations were recorded on the general behavior of the patient, signs of toxicity, and changes in the symptoms and signs of failure.

The two compounds were the U.S.P. ouabain and a specimen of crystals of acetyl-strophanthidin.<sup>4</sup> A stock solution of each in 95 per cent alcohol was prepared, 1:1000 for the

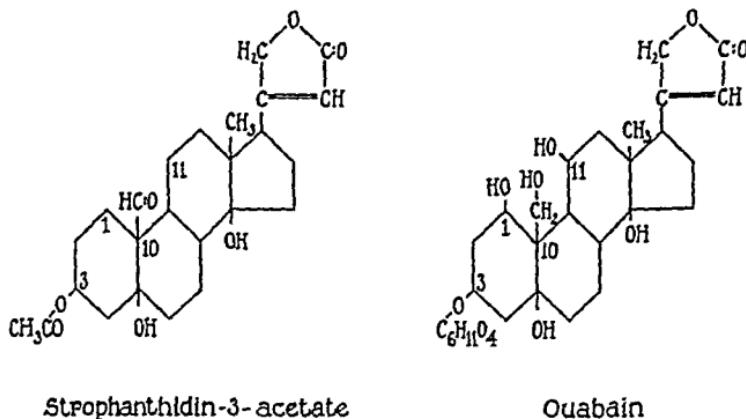
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acetyl-strophanthidin and 1:2000 for the ouabain. The cat unit value of ouabain was taken as 0.1 mgm. per kgm., and of the acetyl-strophanthidin as 0.2 mgm. per kgm. This ratio of potencies seemed to be precise enough for practical purposes inasmuch as the cat unit values for both ouabain and acetyl-strophanthidin vary somewhat with different samples and in different assays. Each dose was 5 cat units<sup>5</sup> given at one time by intravenous injection, the safety of such single doses having been established in previous experiments with



Strophanthidin-3-acetate

Ouabain

FIG. 1

TABLE 1

NAME	SEX	AGE	WEIGHT	DIAGNOSIS*	DEGREE OF HEART FAILURE
			kg.		
Do. St.	F	70	115	A.S., E.H., A.F., Hyperthy.	Advanced
Li. Wi.	F	27	105	R.F., E.H., M.I., M.S., A.F.	Advanced
Be. So.	F	54	106	A.S., R.F., E.H., M.I., M.S., A.F.	Advanced
Es. Ka.	F	50	135	R.F., E.H., M.S., A.F.	Moderate
An. St.	F	49	135	R.F., E.H., M.I., M.S., A.F.	Moderate
Fr. Ko.	F	65	138	Hyper., A.S., E.H., A.F.	Moderate
Gu. Co.	F	48	133	R.F., E.H., M.S., A.F.	Moderate

\* According to the "Nomenclature and Criteria for Diagnosis of Diseases of the Heart" of the New York Heart Association (1942). A.S. (Arteriosclerosis); R.F. (Rheumatic Fever); Hyper. (Hypertension); Hyperthy. (Hyperthyroid); E.H. (Enlarged Heart); Cor. Scler. (Coronary Sclerosis); M.I. (Mitral Insufficiency); M.S. (Mitral Stenosis); A.F. (Atrial Fibrillation).

both materials. The dose of 5 cat units was represented by 1.0 cc. of the above stock solutions, which was diluted to 10 cc. with physiological salt solution for the injection, made in each case over a period of 5 minutes.

RESULTS. There were in all 14 experiments, 7 pairs, each of the 7 patients having received both drugs. The results are charted in figure 2. After the injection, the apex counts were made at intervals of 5 minutes during the first hour,

\* The cat unit is the average fatal dose per kgm. as determined by the method of injection used in the U.S.P. assay of digitalis.

15 minutes during the second hour, 30 minutes during the third hour, at intervals of an hour up to 12 hours after the dose and then 3 times daily. Each point on the two curves represents the average of the ventricular rates for the 7 patients.

The two curves show that 1.0 mgm. of acetyl-strophanthidin produces substantially the same intensity of effect as 0.5 mgm. of ouabain, a slowing of 38 and 42 beats per minute, or 32 and 34 per cent respectively.

The effects develop more rapidly in the case of acetyl-strophanthidin than in the case of ouabain. With acetyl-strophanthidin, approximately 70 per cent of

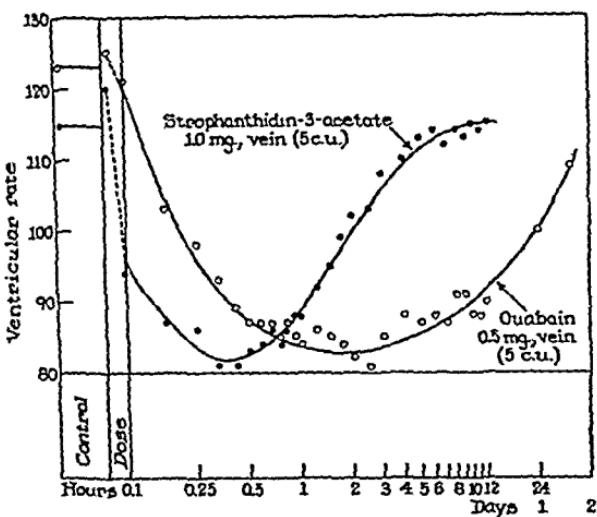


FIG. 2. CURVES OF ACTION OF ACETYL-STROPHANTHIDIN AND OUABAIN, AVERAGE OF 7 PAIRS OF EXPERIMENTS IN 7 PATIENTS WITH AURICULAR FIBRILLATION

Time is charted logarithmically. To conserve space, the control period is contracted into a small section labeled "control"; the horizontal lines indicate the average of the points, which represents the level of the control rate just before the day on which the dose was given. The control count on the day of the dose was sometimes higher and sometimes lower than the general level of the control, as would be expected in the case of the unstable rate in auricular fibrillation. The drug was administered immediately after the count indicated by the first point in the section labeled "dose".

the effect has already developed by the time the injection is completed (about 5 minutes), whereas at this time, only about 10 per cent of the effect of ouabain is in evidence; within 10 minutes after the injection, nearly the full effect (85 per cent) of acetyl-strophanthidin is present, as against about one-half (52 per cent) of the effect in the case of ouabain.

The duration of the effect of acetyl-strophanthidin is much shorter than that of ouabain. In 4 hours, the effects of acetyl-strophanthidin have almost completely disappeared, as against approximately 36 hours in the case of ouabain.

There was no exception in any of the 7 pairs of results, to the general relationship shown in the composite curves of figure 2; namely, that the effect of acetyl-strophanthidin developed and disappeared more quickly than that of ouabain.

In all cases, there was relief from symptoms of failure and from the sense of

palpitation, in association with the cardiac slowing and the development of the full effects of the dose, but these results were temporary because, in the nature of these experiments, the digitalization was not maintained. There were no toxic symptoms.

**COMMENT.** In the vast majority of cases requiring a digitalis action, most of the digitalis materials in common use by oral administration serve the purpose satisfactorily. In these, a delay of a few hours in the development of the full action appears to be of little consequence. There is a relatively small group in which there is considerable urgency. These involve such cases as acute congestive failure with pulmonary edema occurring in advanced hypertensive disease, or after an acute coronary thrombosis, or in a patient with chronic congestive failure after a massive pulmonary infarction. In these cases, the more rapidly acting digitalis glycosides are usually employed by intravenous injection, such as digoxin, lanatoside C, or ouabain. The very urgent cases, however, present special needs, and there is the question whether the curves of action of the above mentioned glycosides fully meet them. There is the fact that in some of these cases, the circulation deteriorates so rapidly that, within a very short period after the onset, the patient is in circulatory collapse and beyond the reach of any form of stimulation. There is also the fact that in these cases the risk of overdigitalization is fairly high, since there is need for heroic measures, and often there is not enough information concerning the amount of digitalis the patient has already had. Inasmuch as the exact dose for any particular patient is unknown, it is necessary to give a safe dose first, and then additional doses until there is evidence of a therapeutic response, or minor toxic effects appear. In the interest of safety, additional doses cannot be given before substantially the full effect of the previous dose has developed. In the case of ouabain, the interval is about an hour or longer. Where several doses of ouabain might be required for a case in extremis, such delay might prove disastrous, and the period of 24 hours or longer for elimination is an additional source of danger in a type of case in which the urgent needs are so apt to lead to intense overdigitalization.

Theoretically, the curve of action of acetyl-strophanthidin is better suited for the treatment of the most urgent problems of acute heart failure. The full effect of an intravenous dose develops in about 10 minutes, producing a digitalis-like stimulation of the heart with approximately the same speed as that of a subcutaneous or intramuscular dose of epinephrine. If additional doses are required, they may be safely given at intervals of 10 to 15 minutes. Should overdigitalization occur in the form of vomiting or an ectopic rhythm, the danger is reduced to a minimum by the high degree of elimination in the next hour or two. After the most serious signs of failure have been brought under control, further digitalization might be procured by the use of the more slowly acting digitalis glycosides.

There is still another type of problem in which the extremely rapid action of acetyl-strophanthidin might be put to good use, namely, to abolish a paroxysm of auricular tachycardia or auricular flutter. It should be noted that the all-or-none principle does not apply to the use of digitalis in the ordinary case of con-

gestive failure. Moderate doses produce some therapeutic effects and larger doses produce greater therapeutic effects. It is otherwise when digitalis is used to abolish a paroxysm of auricular tachycardia or auricular flutter. Here there is only one degree of therapeutic action, namely, that which abolishes the ectopic rhythm, and the drug has been entirely ineffectual unless the dose has been sufficient to achieve that specific end. Here the principle of all-or-none applies. There is also the fact that these ectopic rhythms are sometimes quite resistant, and digitalization must be carried to the point of minor toxicity before the ectopic rhythm will yield. The very rapid development of action and its brief duration in the case of acetyl-strophanthidin would appear to be a favorable combination of properties for such cases.

More extensive experience in the use of acetyl-strophanthidin is necessary in order to establish the most favorable dosage schedules. On the basis of our present results, the following plan may be suggested as safe and effective: an intravenous injection of 0.5 mgm. (2.5 cat units), followed by 0.25 mgm. in 10 minutes, and the latter repeated at intervals of 10 minutes until the therapeutic objective or minor toxic effects are in evidence.

#### SUMMARY AND CONCLUSIONS

1. The curve of action of ouabain was compared with that of acetyl-strophanthidin by intravenous injection in patients with auricular fibrillation and heart failure.
2. The results are based on 14 digitalizations, each with a single dose of 5 cat units, the two drugs being compared in each of 7 patients.
3. Acetyl-strophanthidin exerts a more rapid action in man than any digitalis material in common use; its full effect, after intravenous injection, develops in a period of from 10 to 15 minutes and the effect wears off in a period of about 4 hours, as compared with ouabain in which case about an hour elapses before the full effect develops and about 36 hours before it disappears.
4. The possible utility of acetyl-strophanthidin in extremely urgent cases of acute failure with pulmonary edema, and for the purpose of terminating a paroxysm of auricular tachycardia or auricular flutter is discussed.
5. A tentative plan of dosage is suggested.

These studies were supported in part by the Digitalis Fund of Cornell University Medical College, which includes contributions from Eli Lilly and Company, Wyeth, Inc., E. R. Squibb & Sons, Varick Pharmacal Co., Schering Corporation, Burroughs Wellcome & Co., Winthrop-Stearns Inc., and the David, Josephine, and Winfield Baird Foundation.

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## STUDIES ON PURIFIED DIGITALIS GLYCOSIDES

### VI. THEVETIN, A GLYCOSIDE WITH UNUSUAL SPEED OF ACTION IN MAN

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The name thevetin was first applied by DeVry (1) in 1863 to a glycosidal material extracted from the nut of a tree belonging to the family Apocynaceae. There was little interest in this material until its investigation was resumed by K. K. Chen and his collaborators. In a series of publications since 1933 (2-7), they reviewed the few earlier studies. They pointed out the lack of uniformity in the results regarding constitution and composition, and described the isolation of a pure water soluble crystalline glycoside to which they also applied the name thevetin.

The structural formula of thevetin was determined by Tschesche (8), and its comparison with digitoxin described by Tschesche and Bohle (9), and by Chen, Anderson, and Robbins (10). Figure 1 shows the structure of the aglycone. Thevetigenin is an epimer of digitoxigenin, from which it differs only in the steric arrangement at carbon atom 3; the OH on C<sub>3</sub> and CH<sub>3</sub> on C<sub>10</sub> are in the cis-form in the former, and trans-form in the latter. The glycoside on hydrolysis yields two molecules of glucose and one molecule of a methyl ether sugar which is believed to be digitalose.

In the pharmacological studies of Chen and his collaborators, it was found that thevetin exerts a typical digitalis-like action in the frog, systolic standstill of the frog's heart, and in the cat, typical T-wave changes, impairment of A-V conduction, and rhythm disorders. The cat unit potency of different specimens shows some differences, 1.24 mgm. per kgm. for one material, and 0.92 mgm. per kgm. for a recrystallized material. They found that in animals it also produces vomiting and diarrhea. They suggested that thevetin may be more actively emetic than ouabain, 30 per cent as against 50 per cent of the lethal dose causing vomiting. This difference may be only apparent, resulting from the fact that thevetin is more rapidly eliminated; in such a case, the lethal dose of the two drugs by slow intravenous injection may be relatively further apart than the emetic doses determined by a single intravenous injection.

They also published reports on the use of thevetin in humans, in normal individuals, in patients with heart disease, in patients with congestive failure, and

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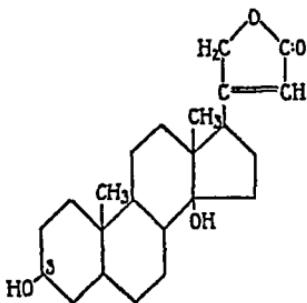
<sup>2</sup> David, Josephine, and Winfield Baird Foundation Fellow, Montreal, Canada.

<sup>3</sup> The authors are indebted to Miss Harriet Zimmerman and Miss Jenny Oppenheim for faithful assistance in these studies.

in patients with the rapid sinus rhythm of thyrotoxicosis. They obtained evidence of typical digitalis action in man after intravenous and oral administration. They encountered a high incidence of gastrointestinal effects, up to 40 per cent after oral doses of the order of 7 cat units or less, given over a period of 4 or 5 days. Cramps and diarrhea were more common than anorexia, nausea, or vomiting. Cramps and diarrhea occurred with a dose as small as 0.5 cat unit 3 times daily.

In cats, they obtained evidence suggesting that the persistence of action was similar to that of ouabain. A study of persistence of action of thevetin in the pigeon and cat by Haag and Pennington (11) showed it to be eliminated with unusual speed, namely, virtually complete in 3 hours in the pigeon, and in about 24 hours in the cat.

The foregoing observations indicated that thevetin possesses unusual properties which might prove to be of special therapeutic interest. Accordingly, the



Thevetigenin  
(Digitoxigenin)

FIG. 1. STRUCTURAL FORMULA OF THEVETIGENIN

It differs from digitoxigenin only in the steric arrangement of the OH group at carbon atom 3.

present study of thevetin action in man was undertaken to throw further light on the curve of its action and its absorption from the gastrointestinal tract.

**METHOD.** The experiments were performed in patients with auricular fibrillation and varying degrees of heart failure. They were selected from a group of 1500 patients in active attendance at our cardiac clinics on the basis of the facts that when without digitalis, their apex rates were rapid, and that they were subject to a degree of failure well within the limits of safety for their use as experimental subjects. The characteristics of the 8 patients used in this study are summarized in table 1.

The method was substantially similar to that used in previous studies (12). The subject who had not been receiving digitalis for several weeks was put to bed in the hospital. The ventricular rate was counted at the apex for one minute, several times daily, under conditions of quiet and rest. This was done during a preliminary control period of a week or longer in order to establish the level of the resting rate without drug. A dose of thevetin was then administered and similar counts were made at suitable intervals until the maximum effect was observed, and at less frequent intervals until the effects disappeared. Several days after the return of the rate to the control level, the subject was ready for another

experiment which was carried out in a similar manner. It was the plan to compare the effects of oral and intravenous doses in one and the same patient in as many experiments as possible. Observations were recorded on the general behavior of the patient, signs of toxicity, and changes in the symptoms and signs of heart failure.

The specimen of thevetin crystals<sup>4</sup> used in this study was assayed in our laboratory and showed a cat unit<sup>5</sup> value of 1.26 mgm. per kgm. For intravenous injection and for oral administration in man, a solution of 1:1000 in 20 per cent alcohol was prepared.

There were in all 15 courses of digitalization with thevetin, 9 by intravenous injection and 6 by oral administration. Four patients received the drug at one time by the intravenous route and at another time by the oral route. The single intravenous doses were 3, 4, and 6 cat units. The single oral doses were 10, 12, 20, and 30 cat units. The intravenous injections were made during periods of from 2 to 5 minutes, and the oral doses were administered on an empty stomach. All experiments were started between 7 and 9 A.M. and breakfast was omitted.

**RESULTS.** The responses to thevetin after intravenous and oral administration were charted and are shown in figures<sup>6</sup> 2 to 9. Time is charted

TABLE 1

NAME	SEX	AGE	WEIGHT lb.	DIAGNOSIS*	DEGREE OF HEART FAILURE
Es. Ka.	F	50	134	R.F., E.H., M.S., A.F.	Moderate
Fr. Ko.	F	65	138	A.S., Hyper., E.H., A.F.	Advanced
Gu. Co.	F	48	133	R.F., E.H., M.S., A.F.	Moderate
An. Pe.	M	62	183	A.S., Hyper., E.H., A.F.	Advanced
Al. Pa.	M	21	160	R.F., E.H., M.I., M.S., A.F.	Moderate
Je. Ma.	F	36	146	R.F., E.H., M.S., A.F.	Moderate
An. Kr.	F	70	153	A.S., E.H., Cor. Scler., A.F.	Moderate
El. Pu.	F	42	136	R.F., E.H., M.I., M.S., A.F.	Moderate

\* According to "Nomenclature and Criteria for Diagnosis of Diseases of the Heart" of the New York Heart Association (1942). A.S. (Arteriosclerosis); R.F. (Rheumatic Fever); Hyper. (Hypertension); E.H. (Enlarged Heart); Cor. Scler. (Coronary Sclerosis); M.I. (Mitral Insufficiency); M.S. (Mitral Stenosis); A.F. (Auricular Fibrillation).

logarithmically. To conserve space, the control period is contracted into a small section labelled "control," and only the last few days of the period are charted; the points indicate the average rates for each day, and the horizontal line the average of the points, which represents the level of the control rate just before the day on which the dose was given. The control count on the day of the dose was sometimes higher and sometimes lower than the general level of the control, as would be expected in the case of the unstable rate in auricular fibrillation. The drug was administered immediately after the count indicated by the first point in the section labelled "dose."

<sup>4</sup> We are indebted to Dr. K. K. Chen of The Lilly Research Laboratories for the supply of thevetin.

<sup>5</sup> The cat unit is the average fatal dose per kgm. as determined by the method of injection used in the U.S.P. assay of digitalis.

<sup>6</sup> In the figures, the number in parenthesis after the symbol refers to the place in the series of experiments with one or another digitalis material, in which the patient served as a subject.

It may be seen that an intravenous dose of thevetin, from 3 to 6 cat units, slows the apex rate in patients with auricular fibrillation, but unlike the digitalis glycosides in common use, the effect develops with extraordinary speed,

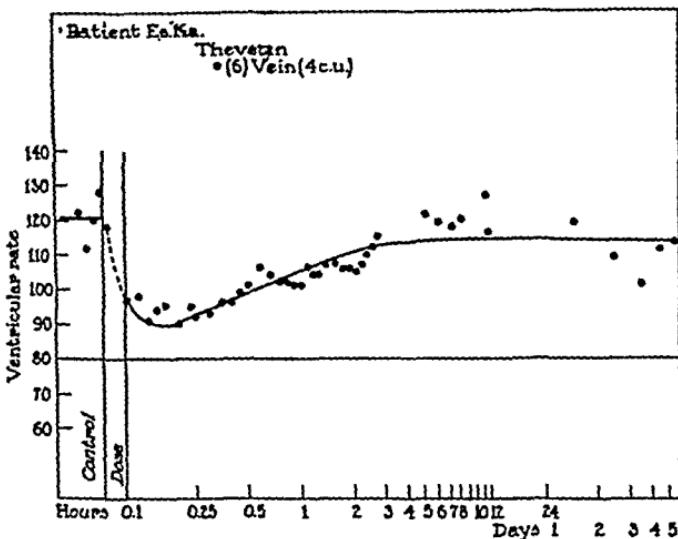


FIG. 2. EFFECT OF INTRAVENOUS DOSE OF THEVETIN ON APEX RATE IN PATIENT WITH AURICULAR FIBRILLATION

Note that the effect was fully developed in about 8 minutes. The rate declined from 118 to 90 per minute. The rate returned to the control level in 2 to 3 hours.

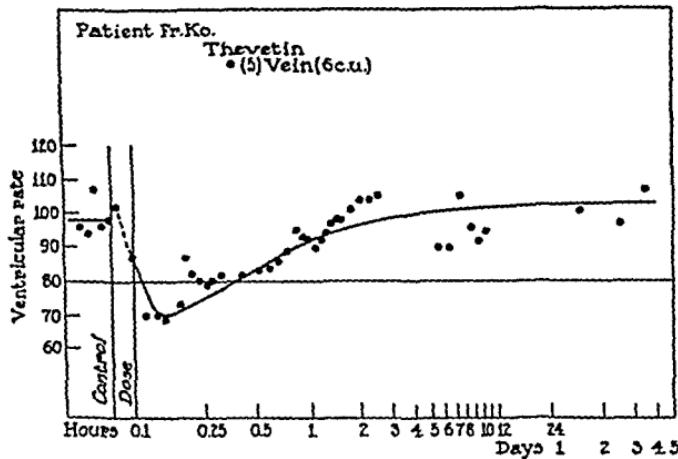


FIG. 3. EFFECT OF INTRAVENOUS DOSE OF THEVETIN ON APEX RATE IN PATIENT WITH AURICULAR FIBRILLATION

The rate declined from about 102 to 70 per minute in about 7 minutes, and returned to the control level in about 2 hours.

the maximum slowing being in evidence in most instances in a period of about 6 minutes after the beginning of the injection. The duration of the effect is very brief. After the maximum slowing, the rate begins to accelerate almost at once

and within about 2 to 3 hours little or none of the slowing remains. In these respects, it resembles some of the genins (13), and some of the esters of strophanthidin (14).

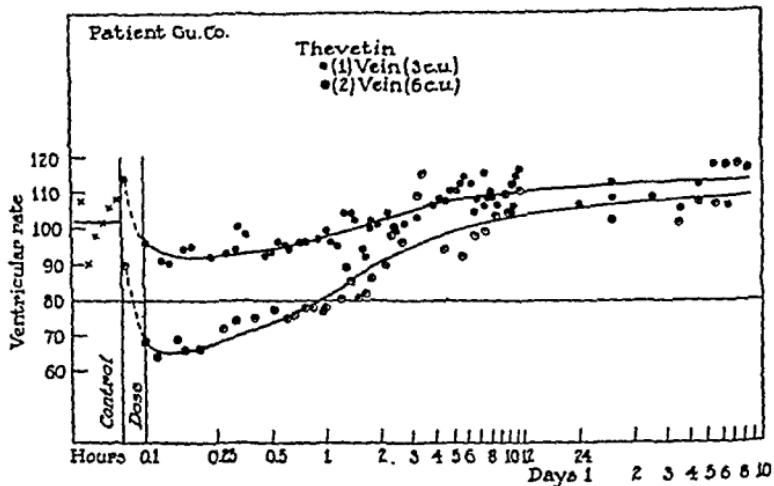


FIG. 4. EFFECT OF INTRAVENOUS DOSES OF THEVETIN ON APEX RATE IN PATIENT WITH AURICULAR FIBRILLATION

As in others, the full effect developed in a period of about 6 or 7 minutes, and disappeared in about 2 to 3 hours. Note the greater sensitivity of the rapid heart to the slowing action of a digitalis material. The dose of 3 cat units of thevetin given at the time when the initial rate was 114 per minute, produced 19.3 per cent slowing, and the dose of 6 cat units given at an initial rate of 90 per minute produced 27.8 per cent slowing; a 44 per cent greater slowing for the 100 per cent greater dose.

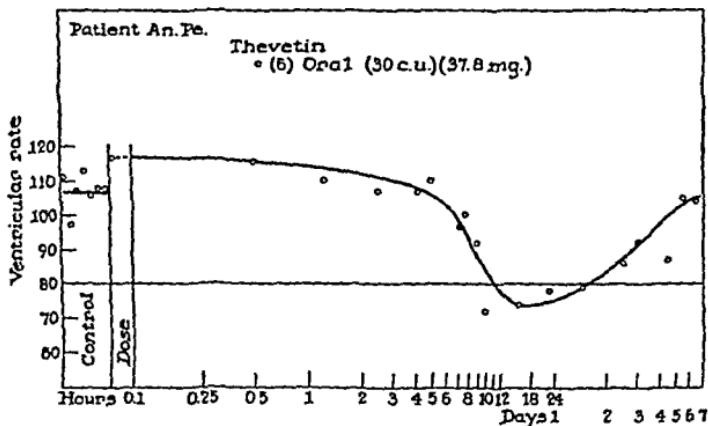


FIG. 5. EFFECT OF ORAL DOSE OF 30 CAT UNITS OF THEVETIN ON APEX RATE IN PATIENT WITH AURICULAR FIBRILLATION

Note the delay of about 7 hours before a perceptible effect, and return to control level in about 4 to 5 days. There were no toxic effects in this case.

The absorption of thevetin from the gastrointestinal tract is slow and variable. A single oral dose of 10 cat units produced no cardiac effects in one case (fig. 8), and double the dose produced some ventricular slowing which began in about 5

hours and disappeared in about 18 hours. In another case (fig. 7), an oral dose of 20 cat units of thevetin produced approximately the same degree of slowing

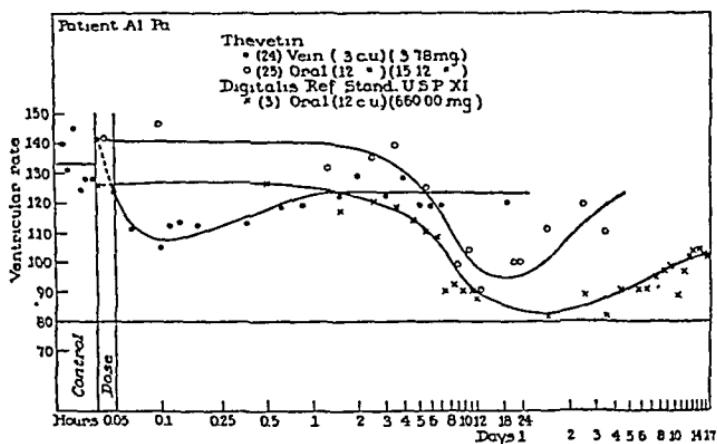


FIG. 6. As in others, note that the full effect of an intravenous dose of thevetin develops in about 5 or 6 minutes and disappears in about 2 hours. Note the greater effect and longer duration of action of 12 cat units of digitalis than of 12 cat units of thevetin by oral administration, 36 per cent slowing as against 29 per cent slowing. The difference in potencies is actually greater than these indicate because the digitalis was operating in a slower or more resistant range of heart rates.

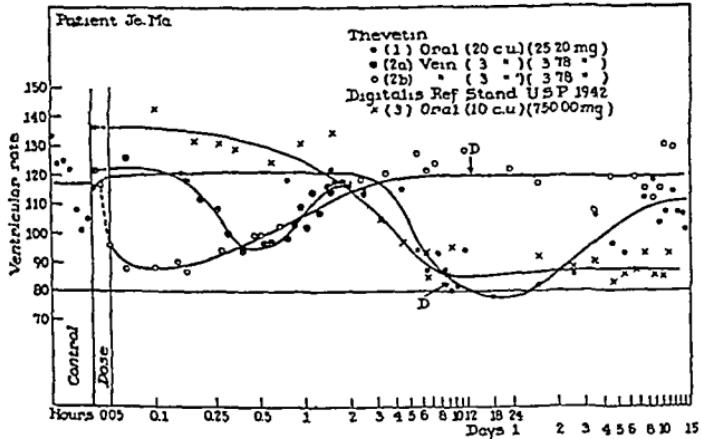


FIG. 7. Curve 2b represents a dose of 3 cat units of thevetin given intravenously at the point where the curve 2a ends; two doses of 3 cat units each were given with an interval of 2 hours. The full effect of the first intravenous dose of 3 cat units developed in about 20 minutes and disappeared in about 1.5 hours; the second dose produced the full effect in about 4 minutes and the effect wore off in about 3 hours; there is indication of slight cumulation. Note the diarrhea (D) about 8 hours after the second intravenous dose. In the case of the oral doses, 20 cat units of thevetin produced essentially the same change (33 per cent slowing) as 10 cat units of digitalis (38 per cent slowing). Note the diarrhea about 8 hours after the oral dose of thevetin.

as 10 cat units of digitalis leaf, and the effect, although relatively fleeting, took about 4 days to wear off. In one case (fig. 5), an oral dose of 30 cat units slowed the apex rate from about 115 to 75 per minute, the effect beginning in about 7

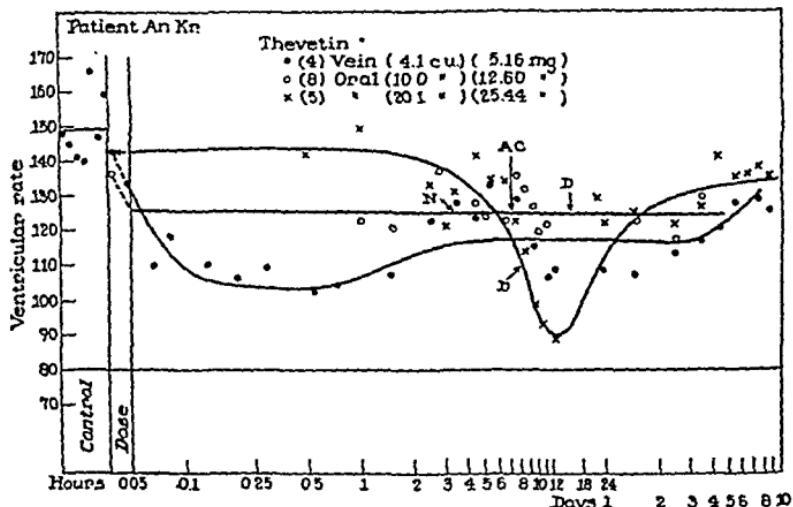


FIG. 8. The intravenous dose produced its full effect in about 6 minutes, and the effect disappeared in about 2 to 3 hours. An oral dose of 10 cats units produced no cardiac slowing, but caused nausea (N), abdominal cramps (AC), and diarrhea (D). Double the dose caused a brief period of slowing, and also produced diarrhea.

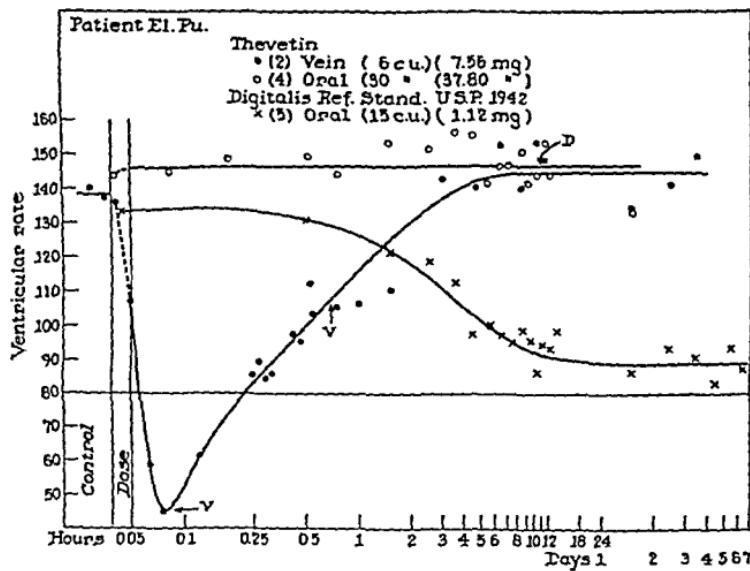


FIG. 9. The intravenous dose of 6 cat units of thevetin slowed the apex rate from 138 to 45 in about 4 minutes, and caused vomiting (V) by systemic action; the effect wore off in about 3 hours. Note the difference in the absorption of thevetin and digitalis; an oral dose of 30 cat units of thevetin produced diarrhea (D) by local action, and no signs of absorption (absence of cardiac slowing), while digitalis in one-half the dose produced the typical effect on the heart rate.

hours after the dose and wearing off in about 4 to 5 days. In another case (fig. 9), a similar oral dose of 30 cat units produced no slowing.

The most striking results were obtained in the case charted in figure 9. An intravenous dose of 6 cat units slowed the apex rate from about 138 to 45 per minute within a period of about 4 minutes after the beginning of the injection. The rate began to rise fairly promptly and reached the original high level in about 2 hours. An oral dose of 30 cat units produced no effects, although this patient had no difficulty in absorbing digitalis; an oral dose of only 15 cat units of digitalis leaf produced the expected results, slowing the rate from about 135 to 85 in a period of about 6 hours.

It is noteworthy that the duration of the effect, when one is produced, after the large oral doses is considerably longer than after the intravenous doses. This may be due, in part, to protracted absorption; there is also the possibility of a conversion into a compound with greater persistence of action.

There was no evidence of toxic rhythms in any of these experiments.

In the matter of gastrointestinal effects, our experience was similar to that of Chen and his collaborators. After 9 intravenous doses in 7 patients, vomiting occurred in one, following a dose of 6 cat units. There was not a single instance of vomiting after 6 oral doses in 5 patients. On the other hand, the oral doses frequently caused diarrhea. This was present in 3 of 5 patients after oral doses of 10, 20, and 30 cat units. It was associated with nausea and abdominal cramps in one of these cases. It appeared in about 8 hours. The fact that it sometimes occurred in the absence of a cardiac effect, indicates that it is probably a local action, although a systemic action of thevetin may also cause diarrhea since it occurred in one case after an intravenous dose of 6 cat units. The frequency of diarrhea distinguishes the experience with thevetin from that with digitalis which usually causes vomiting after large oral doses, and only rarely diarrhea in man. The reason for the differences in the relative emetic and diarrheal activities of digitalis and thevetin is not clear, and the problem is in need of further investigation.

#### SUMMARY AND CONCLUSIONS

1. The speed of action of thevetin and its absorption from the gastrointestinal tract were investigated in 8 selected patients with auricular fibrillation and heart failure. There were 15 courses of digitalization by means of thevetin, and in several instances, the effects of the oral and intravenous doses were compared in the same patient.
2. Thevetin is slowly and irregularly absorbed from the gastrointestinal tract. It is much less effectively absorbed than digitalis leaf.
3. Large oral doses of thevetin frequently cause diarrhea by local action and much less frequently vomiting, in contrast to digitalis after which vomiting from the local action of large oral doses is frequent and diarrhea relatively rare.
4. An intravenous injection of thevetin produces digitalis-like effects on the heart more rapidly than any glycoside of the digitalis series known at the present time. The full effects develop in a period of about 6 minutes after the beginning of the injection.
5. The duration of action of thevetin is very brief. The effects of a fully dig-

italizing intravenous dose disappear almost completely in a period of from 2 to 3 hours. This is more rapid than for any glycoside of the digitalis series known at the present time.

6. The combination of properties, extremely rapid development of action and rapid elimination, suggests interesting possibilities for the use of thevetin by intravenous injection for the treatment of acute heart failure with pulmonary edema, critical conditions in which effective digitalization in a matter of minutes rather than hours may prove decisive. Since in such cases, the danger of overdigitalization is relatively great, the rapid excretion of thevetin provides an important factor of safety.

These studies were supported in part by the Digitalis Fund of Cornell University Medical College, which includes contributions from Eli Lilly and Company, Wyeth, Inc., E. R. Squibb & Sons, Varick Pharmacal Co., Winthrop-Stearns Inc., Schering Corporation, Burroughs Wellcome & Co., and the David, Josephine, and Winfield Baird Foundation.

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# A STUDY OF THE PHARMACOLOGICAL RELATIONSHIP BETWEEN DIGITOXIN AND HISTAMINE<sup>1</sup>

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In previous experiments we showed that digitoxin decreased the histamine output of isolated rabbit and cat hearts (1). This observation leaves incompletely answered the question as to whether there is a pharmacological relationship between digitoxin and histamine. As an approach to this problem three groups of toxicity experiments were carried out: 1. We measured the blood histamine of rabbits during acute digitoxin poisoning; 2. We determined the effects of small amounts of histamine on the toxicity of digitoxin on isolated heart preparations; 3. In order to ascertain the type of joint toxicity produced by the two substances, we carried out acute toxicity studies on normal guinea pigs and determined the LD<sub>50</sub> of histamine and digitoxin given separately, and simultaneously in various proportions.

**MATERIALS AND METHODS.** Seven normal rabbits which were fasted for 18 to 24 hours were injected intravenously with 1 mgm./kgm. of digitoxin. The digitoxin solution was freshly prepared from a stock solution containing 5 mgm. of digitoxin per cc. of 95 per cent ethanol. For the injection of digitoxin the appropriate dose (1 mgm./kgm.) of this alcoholic solution was diluted with 0.9 per cent NaCl to a final volume of 5 cc. The same amount of alcohol (0.2 cc./kgm.), in saline mixture containing no digitoxin, was injected into 3 rabbits. Blood samples for histamine assay were obtained by cardiac puncture at the time intervals indicated in table I. Histamine was measured in trichloroacetic acid extracts of blood by the method of Barsoum and Gaddum (2).

Perfusion experiments were performed on the isolated hearts of rabbits, cats, guinea pigs and rats. The hearts were perfused by the method described previously (1). After a lapse of time varying from 10 to 20 minutes, during which the activity of the heart and the rate of perfusion became constant, separate or simultaneous perfusions with digitoxin (1:40,000) and histamine dihydrochloride (1:1,000,000) in Ringer-Locke solution were commenced. In rabbit, cat and guinea pig heart perfusions the time which passed between the introduction of digitoxin or digitoxin plus histamine and complete heart arrest was noted. The volume of the perfusate was measured for time intervals as given in figure 2. The more resistant rat hearts were allowed to beat for 1 hour with separate and simultaneous perfusions. All hearts of each species were beating at the end of 1 hour in control experiments in which only histamine dihydrochloride (1:1,000,000) was perfused. The hearts were weighed at the end of each experiment to enable calculation of the volume of drug solution perfused per gram of heart.

The toxicity of digitoxin and histamine dihydrochloride in guinea pigs was measured as follows. The guinea pigs, weighing from 200 to 400 grams, were fasted for 24 hours and then injected intraperitoneally with separate and combined solutions of the drugs. The LD<sub>50</sub> of each drug was first determined. To reduce the amount of alcohol injected, a different

<sup>1</sup> This work was supported by grants from the Life Insurance Medical Research Fund and the Office of Naval Research N6ori-20, Task Order #11.

<sup>2</sup> Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

stock solution of digitoxin containing 10 mgm. per cc. of 95 per cent ethanol was used. For injection 2 cc. of this solution was diluted to 100 cc. with 0.9 per cent NaCl to give a final concentration of 0.2 mgm. digitoxin per cc. The amount of alcohol present did not produce toxic symptoms. The solution used for determining the LD<sub>50</sub> of histamine contained 0.5 mgm. of histamine dihydrochloride per cc. of 0.9 per cent NaCl. Toxicity measurements on the drugs combined were carried out using solutions of digitoxin and histamine dihydrochloride in the following ratios of their LD<sub>50</sub>'s, 1:3, 1:1, and 3:1. For each LD<sub>50</sub> three to five groups of 6 animals each were used. In calculating the LD<sub>50</sub> the method of Behrens (3) was applied.

**RESULTS.** The results of the effect of digitoxin and alcohol on the blood histamine of rabbits is presented in table I. While the control blood histamine level showed individual variation, in 6 or 7 animals there was a rapid decrease in the blood histamine following the injection of digitoxin. In animal num-

TABLE I

*The effect of intravenous digitoxin (1 mgm./kgm.) and 95 per cent ethanol (0.2 cc./kgm.) on the blood histamine level of rabbits*

NO. OF ANIMAL	BLOOD HISTAMINE (GAMMA PER GRAM BLOOD)			
	Control	10 minutes	30 minutes	Final sample
1	1.81	1.24	1.06	1.5 (45*)
2	2.8	0.83	0.6	1.04 (51*)
3	1.02	0.63	0.35	0.94 (40*)
4	1.33	0.97	0.59	0.78 (65*)
5	0.8	1.0	0.95	0.57 (50*)
6	2.8	2.0	1.5	1.3 (40*)
7	1.73	0.71	1.0	1.15 (65*)
8†	0.84	0.79	0.76	0.81 (45*)
9†	1.35	1.38	1.36	1.28 (45*)
10†	0.6	0.56	0.62	0.70 (45*)

\* Time of removal of last sample. Animals 1 to 7 in a moribund state.

† These animals received no digitoxin, only 0.2 cc. alcohol per kgm.

ber 5 the decrease occurred 50 minutes after the injection. Some of the rabbits exhibited a terminal increase. In the control animals which received alcohol no significant change in blood histamine could be observed. The action of digitoxin in lowering the blood histamine is similar to its effect on the histamine content of the perfusate of isolated rabbit hearts (1).

A summary of perfusion experiments with digitoxin and histamine is presented in table II. Since the volume of perfusate is known, the amount of histamine dihydrochloride and digitoxin brought in contact with the heart muscle can be calculated. The results of such calculations are presented in table II. The first column indicates the average amounts of digitoxin (gamma per gram of heart) which were sufficient to cause arrest of rabbit, cat and guinea pig isolated hearts. If digitoxin and histamine dihydrochloride were perfused simultaneously, the hearts were killed by much less digitoxin (second column). As shown in the third column, much larger amounts of histamine could be introduced into the

hearts in the course of 1 hour perfusions without exerting a lethal action. Rat hearts proved to be very resistant to digitoxin even with the addition of histamine.

TABLE II

*Absolute amounts of digitoxin and histamine dihydrochloride perfused into isolated hearts at concentrations of 1:40,000 and 1:1,000,000 respectively*

NO. OF ANIMALS USED	AVG. AMT. OF DIGITOXIN CAUSING HEART ARREST	AVG. AMOUNTS OF DIGITOXIN AND HISTAMINE DIHCl CAUSING HEART ARREST WHEN PERFUSED SIMULTANEOUSLY		AVG. AMOUNT OF HISTAMINE DIHCl PERFUSED WITHOUT DIGITOXIN IN 1 HOUR
		Digitoxin	Histamine	
15 rabbits.....	gamma/gram ht.	gamma/gram ht.	gamma/gram ht.	gamma/gram ht.
15 rabbits.....	369	135	5.4	71
17 cats.....	495	285	11.4	124
13 guinea pigs.....	900	361	14.5	233
13 rats.....	1295*	3052*	274*	246

\* This amount was introduced in the course of 1 hour without causing heart arrest.

### EFFECT OF HISTAMINE DIHYDROCHLORIDE (1:1,000,000) ON THE PERfusion RATE OF ISOLATED HEARTS

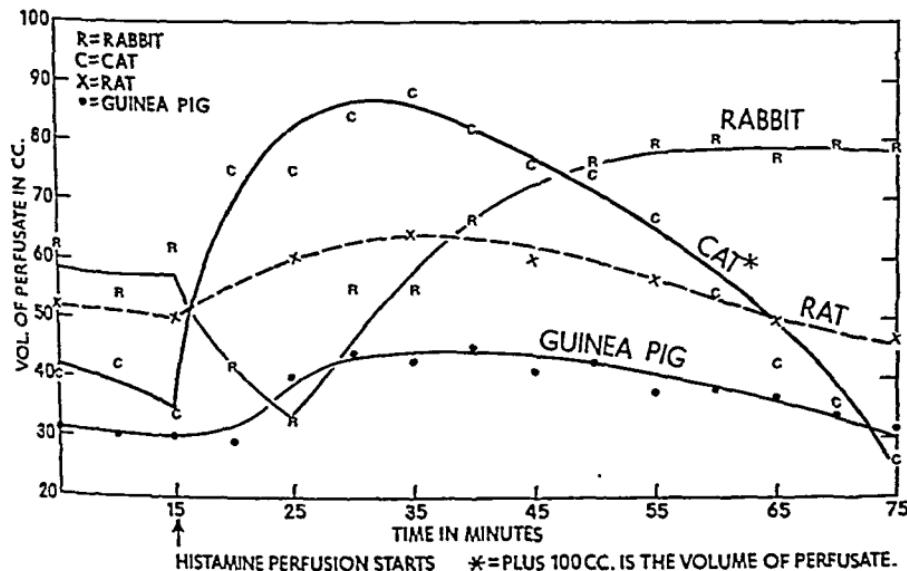


FIG. I. The symbols indicate volumes of perfusate collected in 5 minute intervals in case of rabbit, cat and guinea pig hearts, and in 10 minutes for rat hearts.

In control experiments with digitoxin and histamine dihydrochloride alone it was found that histamine affected the perfusion rates of the isolated hearts. A temporary decrease in perfusion flow, indicative of a coronary constriction, was observed in rabbit hearts while cat, guinea pig and rat hearts exhibited a transient increase in perfusion rate (fig. I). A transitory increase in heart rate and

contractility occurred with rabbit, cat and guinea pig hearts during histamine perfusion but rat hearts were little affected. Digitoxin in the concentration used

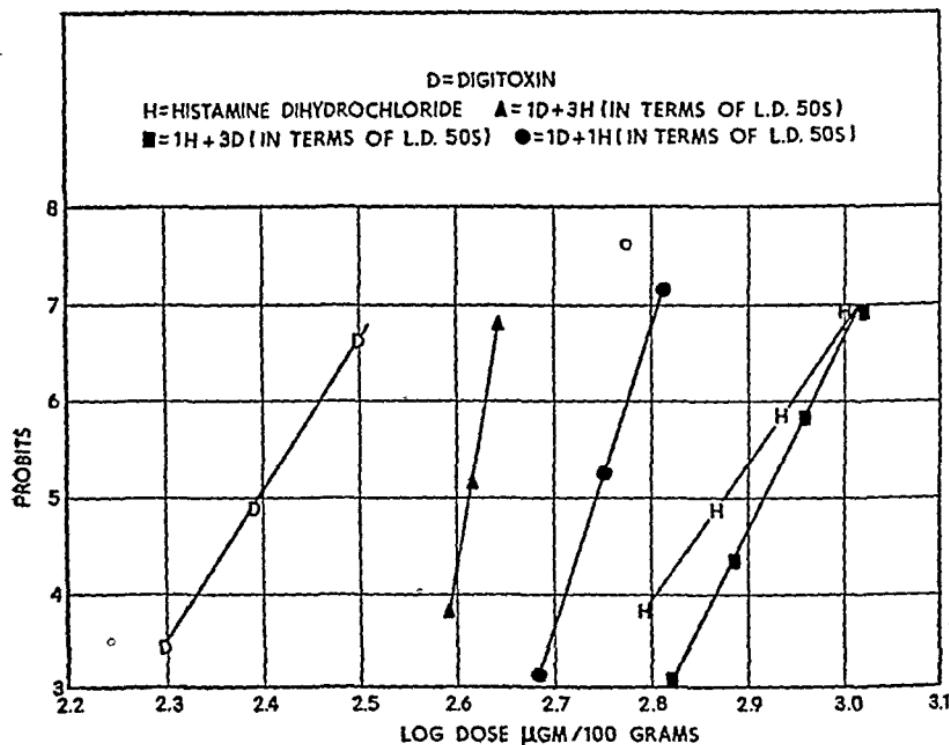


FIG. II. DOSE-MORTALITY CURVES OF DIGITOXIN AND HISTAMINE DIHYDROCHLORIDE  
Log dose of mixtures is that of total dose ( $H + D$ ). The guinea pigs were injected intraperitoneally.

TABLE III

The  $LD_{50}$ s of digitoxin and histamine dihydrochloride alone and in mixture

HISTAMINE DIHYDROCHLORIDE*	DIGITOXIN*
mgm. per 100 grams	mgm. per 100 grams
0.000	0.256
0.771	0.000
0.206	0.204
0.313	0.104
0.485	0.054

\* Injected intraperitoneally into guinea pigs.

(1:40,000) had no measurable effect on the rate of perfusion during the survival time of the isolated hearts.

A graphic representation of the toxicity experiments in guinea pigs is given in figure II wherein the dosage-mortality curves of digitoxin and histamine dihydrochloride separately and in three different combinations are presented. Mortality studies with histamine and digitoxin reveal that mixtures (see table III)

of the drugs produce a higher mortality rate than can be accounted for by a joint toxicity which is due to an independent and similar action of the two drugs. For instance, the LD<sub>50</sub> of histamine dihydrochloride is 0.77 mgm. per 100 grams and the LD<sub>50</sub> of digitoxin is 0.256 mgm. per 100 grams. In a mixture of 1 histamine dihydrochloride to 1 digitoxin in terms of LD<sub>50</sub>'s, the amount of each drug in the LD<sub>50</sub> dose is 0.313 mgm. per 100 grams and 0.104 mgm. per 100 grams, respectively. Furthermore, the dosage-mortality curves of the combinations of digitoxin and histamine appear to be those of different drugs. When the amounts of histamine dihydrochloride and digitoxin present in LD<sub>50</sub> doses

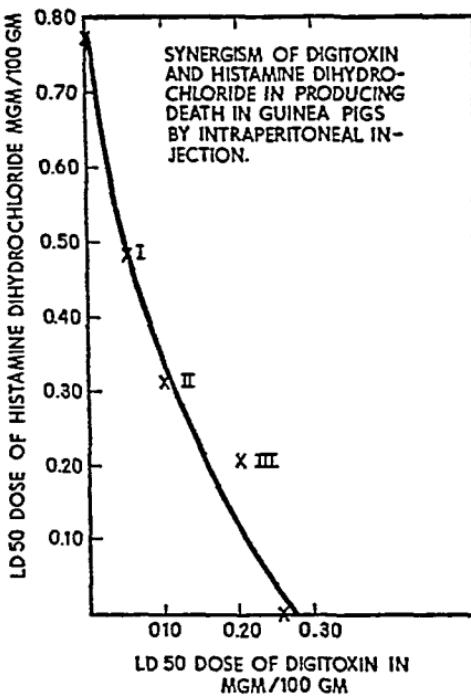


FIG. III. The points represent the LD<sub>50</sub> doses alone and in mixtures (I = 3H:1D; II = 1H:1D; III = 1H:3D; in terms of LD<sub>50</sub>'s).

(alone and in mixture) are plotted against each other, a concave curve is obtained (fig. III). The curve suggests that in mixture the 2 drugs show a synergism in killing guinea pigs.

**DISCUSSION.** The material presented in this paper offers further evidence that there is a pharmacological relationship between digitoxin and histamine. It was shown that digitoxin exerts an effect on rabbit blood histamine similar to its effect on the perfusates of isolated rabbit hearts. The decrease in blood histamine observed was followed usually by a final tendency to increase. This latter effect may be due to a compensatory mechanism of the total organism or to anoxia, since the poisoned animals showed a terminal dyspnea and cyanosis. The second explanation is suggested by the results of Eichler and co-

workers (4) who reported that exposure of cats to an atmosphere of 10 per cent oxygen raised the plasma histamine concentration.

In the presence of sublethal quantities of histamine dihydrochloride rabbit, cat and guinea pig isolated hearts behaved similarly and were arrested by much smaller amounts of digitoxin. A joint toxicity study on guinea pigs demonstrated a synergism between the two drugs, that is to say, the total reaction was greater than could be due to a summation of the individual reactions. In figure III only point III falls somewhat off the curve. If we consider this value as significant, the joint lethal action of the two substances in mixture III is additive, while in the other ratios the effects of histamine and digitoxin were more than additive. A quantitative determination of the degree of synergism was not the task of the present investigation, however, the given evaluation of the data offers sufficient evidence to show that histamine and digitoxin potentiate each other in their lethal action. This potentiation was more pronounced in isolated guinea pig hearts than in the whole animal. The small amount of histamine dihydrochloride used in the perfusion experiments had a considerable effect on the coronary flow as measured by the volume of perfusate. With the exception of the rabbit heart, there was an initial increase in volume of perfusate followed by a reversal. The rabbit heart behaved in an opposite manner. Dale and Laidlaw (cf. (5)) found a dilating effect of histamine in cat hearts. Andrus and Wilcox (6) found that the coronary outflow of the guinea pig heart was decreased when histamine was added to the perfusion fluid. Wilcox and Seegal (7) later stated that the effect of histamine on the guinea pig coronary flow was dependent on the dose. This is in agreement with our findings. It might be assumed that the initial effect is due to the small concentration of histamine in the heart, whereas the reversal which follows is a consequence of a gradual accumulation of histamine in the heart muscle during the perfusion. The species differences demonstrated rule out the possibility that changes in coronary flow could be responsible for the potentiation observed in the isolated heart experiments.

#### SUMMARY

1. Following the intravenous injection of digitoxin (1 mgm./kgm.) into rabbits the blood histamine level decreased.
2. Histamine dihydrochloride (1:1,000,000) greatly potentiated the lethal effect of digitoxin (1:40,000) on the isolated hearts of rabbits, cats and guinea pigs. The hearts of rats were resistant under these experimental conditions.
3. Histamine dihydrochloride (1:1,000,000) exerted a diphasic action on the coronary outflow of isolated hearts. The hearts of cats, guinea pigs and rats exhibited an initial coronary dilatation while a constriction was observed in the case of rabbit hearts.
4. In joint toxicity studies on guinea pigs digitoxin and histamine dihydrochloride acted synergistically in producing death of the animals.

*Acknowledgements.* Our sincere thanks are due to Dr. E. M. K. Geiling and Dr. J. M. Coon for their kind help and for much valuable assistance. We are

also grateful to Eli Lilly and Company, Indianapolis, for generously furnishing the digitoxin used in this study.

*Addendum.* Since this manuscript was submitted for publication, joint action experiments have been carried out on strips of atropinized guinea pig ileum. It was found that 0.2  $\mu$ gm. of histamine greatly augmented the contractile response of the gut to 30  $\mu$ gm. of digitoxin. Again the response was in the nature of a synergism.

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# SYNERGISTIC EFFECT OF CHLORGUANIDE AND SULFADIAZINE AGAINST PLASMODIUM GALLINACEUM IN THE CHICK

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Sulfadiazine was the first compound proven to be a complete causal prophylactic against *Plasmodium gallinaceum* in the chick (1, 2, 3) and has been used by many laboratories, including our own, as the reference drug in prophylactic antimarial screening tests. The procedure employed for such tests in this laboratory has been described in detail in Wiselogle (4, see TEST A-2) and is only briefly summarized here. One-week-old chicks (*Gallus domesticus*) are treated twice daily (b.i.d.) for 4 days, beginning 4 to 5 hours before subcutaneous inoculation with sporozoites of *P. gallinaceum* Brumpt (strain 8A). A drug is considered active if at least 50 per cent of the treated birds show no evidence of infection for 35 days after inoculation (*complete protection*) or if, in at least half of the birds treated, there is a delay in the onset of parasitemia (*partial protection*). Over a period of four years, progressively larger doses of sulfadiazine have been required to give complete protection. In 1944, 0.125 mgm./gram (body weight) b.i.d. completely protected almost all birds tested, whereas in 1947, as much as 0.5 mgm./gram b.i.d. protected less than half of them.

Among the comparatively few compounds, other than the sulfonamides, found to have prophylactic action against *P. gallinaceum* is chlorguanide [1-(*p*-chlorophenyl)-5-isopropylbiguanide].<sup>1</sup> Curd, Davey and Rose (5) have reported complete protection against the infection with this drug. In our A-2 tests, chlorguanide at the maximum tolerated dosage level, 0.03 mgm./gram b.i.d.,<sup>2</sup> delays onset of parasitemia but protects very few birds completely. At toxic levels, though, it has been found to be a true causal prophylactic.

It was considered of interest to determine the effect of simultaneous administration of chlorguanide and sulfadiazine on sporozoite-induced *P. gallinaceum*. In a pilot experiment, chlorguanide at 0.015 mgm./gram b.i.d. plus sulfadiazine at 0.125 mgm./gram b.i.d. completely protected all the birds, while each drug given alone at the same dosage did not give complete protection in any instance. It was decided to extend the observations to see whether the effect was a synergism or a true potentiation.

The results of three prophylactic experiments have been combined in table 1. Chlorguanide, at 0.015 mgm./gram b.i.d., when combined with sulfadiazine, at 0.0078 mgm./gram b.i.d., protected 7 of 10 birds completely. In the remaining

<sup>1</sup> Chlorguanide hydrochloride is the generic designation recognized by the Council on Pharmacy and Chemistry of the American Medical Association for this compound, widely known under a British trade name, "Paludrine hydrochloride." (See J. A. M. A., 136: 251, 1948.)

<sup>2</sup> All chlorguanide doses are given in terms of the monohydrochloride salt.

3, there was a marked delay in the appearance of initial parasitemia. However, when the drugs were given alone, sulfadiazine at 0.5 mgm./gram b.i.d. protected less than half of the birds, and chlorguanide at 0.06 mgm./gram b.i.d., a dosage highly toxic to chicks, protected slightly more than half the birds not dying of drug toxicity. If C represents the minimal dose of chlorguanide which protected half the birds when given alone and S, the minimal dose of sulfadiazine which protected half when given alone, then the minimal effective doses of the drugs

in combination were found to be:  $\frac{C}{4} + \frac{S}{64^+}$ .

The potentiation also occurs in blood-induced *P. gallinaceum* infections in the chick. The drugs have been tested according to our routine A-1 procedure, also

TABLE 1

*The effect of chlorguanide and sulfadiazine given alone and in combination<sup>a</sup> against sporozoite-induced Plasmodium gallinaceum in chicks<sup>b</sup>*

DOSE OF CHLORGUANIDE HYDROCHLORIDE IN MG.M./GRAM B.I.D.	DOSE OF SULFADIAZINE IN MG.M./GRAM B.I.D.									
	None	0.00195	0.0039	0.0078	0.0156	0.03125	0.0625	0.125	0.25	0.5
No. of birds showing no parasitemia for 35 days after inoculation/no. of birds treated										
None	0/30	—	—	—	0/10	0/10	1/10	6/29	6/30	2/10
0.0009375	—	—	—	—	0/10	—	—	—	—	—
0.001875	—	—	—	—	0/9	—	—	—	—	—
0.00375	—	—	—	—	0/10	—	—	—	—	—
0.0075	—	—	—	—	0/10	—	—	—	—	—
0.015	0/20	2/10	2/10	7/10	19/24	15/16	5/5	6/6	—	—
0.03	6/29	—	—	—	—	—	—	—	—	—
0.06	5/9	—	—	—	—	—	—	—	—	—

<sup>a</sup> Serial dilutions were made from stock aqueous solutions of chlorguanide hydrochloride and sulfadiazine. The diluted drugs were combined in proper proportions to give the designated doses.

<sup>b</sup> Drugs were given 4 days b.i.d. beginning 4 to 5 hours before subcutaneous inoculation of birds with sporozoites. Data from three experiments have been combined.

described in Wiselogle (4). In this test the birds are treated twice daily for 4 days, beginning 4 to 5 hours before inoculation with heparinized blood containing  $16 \times 10^6$  parasitized erythrocytes. Parasite counts (parasitized cells per  $10^4$  erythrocytes) are made the morning after the last day of treatment. The minimal effective dose in this test is, by definition, that dose of drug which causes a 75 per cent reduction in mean parasitemia in treated birds as compared with untreated controls. As seen in table 2, where two separate experiments have been summarized, the minimal effective dose of chlorguanide was 0.002 mgm./gram b.i.d. and of sulfadiazine, 0.03 mgm./gram b.i.d. If C represents the minimal effective dose of chlorguanide when given alone and S, the minimal effective dose of sulfadiazine when given alone, then the minimal effective doses of the drugs in combination were found to be:  $\frac{C}{4} + \frac{S}{32}$  or  $\frac{C}{8} + \frac{S}{4}$ . The potentiation,

being limited by the drug present in least dilution, therefore, would be about four-fold.

The subacute toxicity (4-day) in chicks of the drugs in combination, as obtained by the weight-gain method (4, see TEST 1-A), appears to be additive. We are indebted to Dr. Nathan B. Eddy, Principal Pharmacologist, Division of Physiology, National Institute of Health, for toxicity data.

It has also been found from studies on uninfected chicks that the plasma levels of sulfadiazine in individual chicks, as determined by the method of Bratton and

TABLE 2

*The effect of chlorguanide and sulfadiazine given alone and in combination\* against blood-induced Plasmodium gallinaceum in chicks<sup>b</sup>*

DOSE OF CHLORGUANIDE HYDROCHLORIDE IN MG.M./GRAM B.I.D.	EX- PERI- MENT NO.	DOSE OF SULFADIAZINE IN MG.M./GRAM B.I.D.										
		None	0.000234	0.000468	0.0009375	0.00185	0.00375	0.0075	0.015	0.03	0.06	
		Mean parasite count (parasitized cells per 10 <sup>4</sup> erythrocytes) <sup>c</sup>										
None	1	6,480	—	—	—	—	—	—	4,626	3,181	838	77
	2	8,360	—	—	—	—	—	—	7,090	4,347	1,924	304
0.00025	1	—	7,420	7,940	5,900	4,752	3,161	1,523	856	—	—	—
0.0005	1	6,338	3,055	3,205	3,325	728	46	—	—	—	—	—
	2	7,320	—	—	—	—	—	—	—	—	—	—
0.001	1	3,737	517	99	—	—	—	—	—	—	—	—
	2	4,326	—	—	26	24	14	8	11	—	—	—
0.002	1	259	—	—	—	—	—	—	—	—	—	—
	2	236	—	—	—	—	—	—	—	—	—	—
0.004	1	11	—	—	—	—	—	—	—	—	—	—
	2	56	—	—	—	—	—	—	—	—	—	—

\* See footnote a, Table 1.

<sup>b</sup> Drugs were given 4 days b.i.d. beginning 4 to 5 hours before intravenous inoculation of each bird with an inoculum containing  $16 \times 10^6$  parasitized erythrocytes.

<sup>c</sup> Parasite counts were made the morning following the last day of treatment. Each mean represents 10 birds. Bold-faced type indicates mean parasite counts which are at least 75% below the mean parasite count of the corresponding untreated controls.

Marshall (6)<sup>3</sup>, were 33-100 per cent higher in those which received chlorguanide and sulfadiazine simultaneously, than in those which were given sulfadiazine alone (table 3). This increase in sulfadiazine plasma levels, although statistically significant, does not account for the increased rate of protection found when chlorguanide and sulfadiazine are administered concurrently. A contrast of two infected groups will make this evident. When sulfadiazine was administered alone at a dosage of 0.25 mgm./gram b.i.d., it protected less than half the chicks

<sup>3</sup> When both drugs are administered concurrently, the presence of one drug does not interfere with the measurement of the other.

in prophylactic tests (table 1) even though the resulting plasma level was 19.0 mgm. per cent (table 3). When sulfadiazine was administered at a dosage of 0.0156 mgm./gram b.i.d. concurrently with chlorguanide at a dosage of 0.015 mgm./gram b.i.d., 19/24 chicks were completely protected while the sulfadiazine plasma level was 2.8 mgm. per cent (table 3). A similar series of chlorguanide plasma levels (unpublished data), determined according to the method of Spinks and Tottey (7)<sup>a</sup> on pooled samples from 5-10 chicks, showed no consistent differences between chicks receiving concurrent sulfadiazine and chlorguanide and those receiving chlorguanide alone.

Further investigations are now in progress to find whether the potentiation with chlorguanide extends to other sulfonamides and to determine the mode

TABLE 3

*The concentration of sulfadiazine in the plasma of uninfected chicks given sulfadiazine alone and in combination<sup>b</sup> with chlorguanide<sup>c</sup>*

NUMBER OF BIRDS	DOSE OF SULFADIAZINE mgm./gram b.i.d.	DOSE OF CHLORGUANIDE HYDROCHLORIDE mgm./gram b.i.d.	MEAN PLASMA LEVEL OF SULFADIAZINE mgm. %
7	0.0039	0.015	1.3 ± 0.16
7	0.0156	—	2.0 ± 0.23
9	0.0156	0.015	2.8 ± 0.21
10	0.03125	—	3.4 ± 0.5
9	0.03125	0.03	6.5 ± 0.6
5 <sup>c</sup>	0.25	—	19.0
5 <sup>c</sup>	0.25	0.03	27.0

<sup>a</sup> See footnote a, Table 1.

<sup>b</sup> Drugs were given 3½ days b.i.d. Blood samples were drawn 3 hours after last dose.

<sup>c</sup> Samples pooled.

of action of the potentiation. It is suggested that the potentiation of effect achieved with chlorguanide plus sulfadiazine may extend to bacterial infections, including those in which sulfonamides are only partially effective. The application to human malaria is obvious, and experiments are now in progress by other members of the staff to test both the therapeutic and the prophylactic effect of chlorguanide-sulfadiazine combinations against *Plasmodium vivax* in man.

#### SUMMARY

Concurrent administration of chlorguanide and sulfadiazine was examined for prophylactic and suppressive activity against *Plasmodium gallinaceum* infections in the chick. It was found that the two drugs potentiate one another. The effective dose of chlorguanide could be reduced to one-quarter when combined with 3½ to 7½ the effective dose of sulfadiazine. The potentiation could not be

accounted for by the concentrations of the drugs in the blood. The toxicity of the two drugs in combination was additive.

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# A STUDY ON THE COMPARATIVE TOXIC EFFECTS OF CITRIC ACID AND ITS SODIUM SALTS

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Since the introduction of citrated blood for transfusion in 1915 by Lewisohn (1), there has been considerable study as to its value and its disadvantages. In the original work, the relative safety of the procedure was demonstrated first in dogs and then in man. Following this, much was done to improve the technique and thereby reduce the number and severity of the untoward reactions. In recent years under the stimulus of massive transfusions of citrated blood for treatment of extreme shock produced by hemorrhage, there has been much study and speculation as to the occurrence of citrate intoxication (2). However, there apparently have been no comparative studies as to the toxicity of citric acid and its sodium salts as measured by the LD<sub>50</sub>, although anticoagulant acid citrate dextrose solution is U.S.P. The following work was undertaken to determine the LD<sub>50</sub> for citric acid as well as for its sodium salts.

**METHOD.** White mice, albino rats, rabbits and dogs were used as experimental animals. Equal molecular concentrations of citrate in the forms of citric acid, monosodium citrate, disodium citrate, and trisodium citrate were compared. Five hundred and ninety-nine intraperitoneal injections of 0.0477 Molar solutions were made in four hundred and seventy-seven mice weighing between 14 and 28 grams with an average weight of 19 grams. Three hundred and eighty-four intraperitoneal injections of 0.381 Molar solutions were made in two hundred and ninety-nine rats weighing between 85 and 170 grams with an average weight of 120 grams. Three hundred and sixty-one intravenous injections of 0.0119 Molar solutions were made into the tail veins of two hundred and seventy-nine mice weighing between 14.5 and 28.5 grams with an average weight of 22 grams and in these the whole dose was given within a few seconds. One hundred and fifty-eight intravenous injections of 0.477 Molar solutions were made into the lateral ear veins of one hundred rabbits weighing between 1.75 and 2.75 kilograms with an average weight of 1.95 kilograms. Here a constant injection rate of 0.75 cc./minute (0.358 millimoles/minute) was used. The LD<sub>50</sub> was determined with each method of administration for citric acid, monosodium citrate, disodium citrate, and trisodium citrate by the method of Reed and Muench (3).

Eighty intravenous injections of 0.25 Molar solutions were made into the tail veins of eighty mice weighing between 19 and 29 grams with an average weight of 24 grams. Twenty mice were used for each compound. In these experiments the injections were made at a constant rate of 6 cc./minute and continued until the animals died. The individual lethal doses in millimoles per gram of each of the citrate solutions injected were averaged for each twenty animals, and the standard deviations determined.

Under ether anesthesia lumbar cordotomies were performed on seven dogs weighing between 10 and 15 kgm. As soon as the animals recovered from the anesthetic the left femoral artery was cannulated and connected to a mercury manometer using heparin as the anticoagulant. Four animals received trisodium citrate (2.94 grams/kilogram/100 cc.) and the other three were given equal molar quantities of citric acid (2.10 grams/kilogram/100 cc.) intravenously at a constant rate of 0.67 cc./minute until death resulted.

**RESULTS.** The visible responses of mice, rats, rabbits and dogs to toxic doses of citric acid, monosodium citrate, disodium citrate, and trisodium citrate were similar and consisted primarily of increased general activity, hyperpnea, vaso-dilatation of the peripheral vessels, salivation, muscle twitching, clonic and tonic convulsions, cyanosis, Cheyne-Stokes respiration and some deaths. In all of the animals receiving single injections except those injected intraperitoneally with citric acid, if recovery occurred it was apparently complete within a few minutes. These findings are explained adequately by the fact that there is formation of double salts with calcium which do not liberate calcium ions (4).

Since this is intended as a comparative study the doses are expressed in millimoles/kilogram in table I instead of the usual grams/kilogram. Because the molecular concentration of the citrate ion was kept constant, in so far as citrate ion effect is concerned, the LD<sub>50</sub> determinations of the various salts can be compared directly. Citric acid and its sodium salts have the same toxicity when given slowly intravenously to rabbits (see table I). When citric acid was given

TABLE I

*The toxicity of citric acid and its sodium salts in millimoles/kilogram as measured by means of the LD<sub>50</sub>*

ANIMAL	NO.	ROUTE OF ADMINISTRATION	CITRIC ACID	MONO-SODIUM CITRATE	DI-SODIUM CITRATE	TRI-SODIUM CITRATE
Rabbits	158	Intravenous at rate of 0.35S millimoles/minute	1.72	1.76	1.77	1.74
White mice	361	Rapid intravenous	0.22	0.23	0.30	0.66
White mice	599	Intraperitoneally	5.0	7.6	7.5	5.5
Albino rats	384	Intraperitoneally	4.6	6.3	7.3	6.0

intraperitoneally to rats and mice a number of animals died as long as one week after recovery from the immediate toxic effects. Gross post-mortem examinations performed upon many of these animals did not reveal the cause of death. If these compounds are injected rapidly intravenously in mice significant differences in the toxicity are observed (see table I). It would appear that the acid rather than the citrate part of the molecule is the cause of this difference in toxicity.

In those experiments on mice in which intravenous injections of 0.25 Molar solutions were made at a constant rate of six cubic centimeters per minute (1.5 millimoles of the drug per minute) no significant differences in the averages of the individual lethal doses were noted. In the 80 experiments (20 for citric acid and 20 for each of its sodium salts) the average dose necessary to kill all of the animals with the standard deviation was  $2.08 \pm 0.11$ ,  $2.01 \pm 0.09$ ,  $2.21 \pm 0.10$ , and  $2.24 \pm 0.51$  for citric acid, monosodium citrate, disodium citrate and tri-sodium citrate respectively.

In the experiments on dogs in which blood pressures were recorded there was a gradual fall in blood pressure during citric acid injection until near death when the pressure fell precipitously to zero. When sodium citrate was used the blood

pressure remained fairly normal during the injection until just prior to the death of the animal when it fell abruptly to zero.

**DISCUSSION.** There are four criticisms to drawing any conclusions as to the effect of citrated blood for transfusion purposes in man from our results: first, our results were obtained from animals and may not be directly applicable to man on a kgm./kgm. basis; second, these animals were injected with pure drug, whereas, the citrate solution may be altered by the presence of the blood for transfusion; third, the rate of injection in these animals was much more rapid in millimoles/kgm./minute than would ever be given to man in the form of citrated blood; fourth, the patients are not well at the time of transfusion.

Theoretically, ignoring the above criticisms and assuming the LD<sub>50</sub> for man to be about 1.75 millimoles/kgm., or near that for the slow intravenous injection of rabbits, about one liter of anticoagulant acid citrate dextrose solution U.S.P. would have to be given to a 70 kgm. man in a 15 minute interval or less in order to cause the demise of one-half of the patients so treated.

#### SUMMARY

1. The train of symptoms following the administration of citric acid and its sodium salts in toxic quantities appears to be identical with that of calcium ion deficiency consisting of increased general activity, hyperpnea, vaso-dilatation of the peripheral vessels, salivation, muscle twitching, clonic and tonic convulsions, cyanosis, Cheyne-Stokes respirations and some deaths.

2. The LD<sub>50</sub> for citric acid, monosodium citrate, disodium citrate and trisodium citrate in millimoles/kgm. was as follows: intravenous administration in rabbits 1.72, 1.76, 1.77, and 1.74 respectively; for intravenous administration in mice 0.22, 0.23, 0.30, and 0.66 respectively; for intraperitoneal administration in mice 5.0, 7.6, 7.5 and 5.5 respectively; and for intraperitoneal administration in rats 4.6, 6.3, 7.3, and 6.0 respectively. These results suggest that citrate intoxication in massive transfusions does not occur.

We wish to thank Dr. Charles M. Gruber and Dr. Harold W. Jones for their assistance.

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# AN ULTRAVIOLET SPECTROPHOTOMETRIC PROCEDURE FOR THE DETERMINATION OF BARBITURATES

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In a recent paper (1) a procedure for the determination of thiopental (ethyl 1-methyl butyl thiobarbiturate) based on its strong ultraviolet absorption spectra was demonstrated to be highly specific, sensitive, and quantitative. The present report is of a procedure that is applicable to the quantitative determination of barbiturates other than thiobarbiturates. The principle of the method is based on the extraction of the drug by an organic solvent and its removal from the organic solvent by extraction with alkali. The concentration of barbiturate in the alkaline solution is determined by the intensity of the absorption in the ultraviolet.

**EXPERIMENTAL.** For the optical measurements, the Beckman photoelectric quartz spectrophotometer with the standard 10 mm. square quartz absorption cells is used.

In fig. 1 are shown the absorption spectra of some representative barbiturates in 0.5 N sodium hydroxide. These spectra are similar to those found by Elvidge (2) and by Stuckey (3). Each compound is characterized by intense ultraviolet absorption with a maximum at 255 m $\mu$  and a minimum at 235 m $\mu$ . At 255 m $\mu$ , concentration bears a linear relationship to the optical density up to at least 20 micrograms per cc. for each of the barbiturates (fig. 2). Significant optical density readings are obtained with concentrations as low as 2 micrograms per cc. of solution.

*Determination of barbiturates in plasma or whole blood.* By means of a burette, 25 or 50 cc. of redistilled chloroform are run into a separatory funnel. Samples of 0.5 cc. to 5 cc. of blood are added to the chloroform and the separatory funnel is shaken for approximately 3 minutes.<sup>1</sup> After the two phases are allowed to separate, the chloroform is cleared through a dry filter paper to obtain an aliquot of 20 or 40 cc., depending on the initial volume of chloroform. It is now transferred to another separatory funnel and extracted with 5 cc. of 0.5 N sodium hydroxide. The lower chloroform layer is discarded while the alkaline solution, containing the barbiturate as its sodium salt, is run into a test tube and centrifuged to bring down suspended chloroform. Three cc. of the clear alkaline solution are transferred to the absorption cells, and the ultraviolet absorption spectrum determined against a reference blank solution of 0.5 N sodium hydroxide. The optical density of the particular barbiturate at the absorption maximum, 255 m $\mu$  (fig. 1) is used for the estimation of the concentration. The

<sup>1</sup> The addition of a buffer to adjust the pH of the blood was not necessary, since it was found that all the above barbiturates could be extracted quantitatively up to a pH of 7.5.

values for 50 micrograms of barbiturates in 5 cc. of sodium hydroxide range from an optical density of 0.28 for seconal to 0.32 for phenobarbital (fig. 1). A reagent blank is determined by substituting water for blood in this procedure.

*Determination of barbiturates in tissues.* Tissues are prepared for extraction by homogenizing with M/15 phosphate buffer, pH 7, in either a Waring blender or in an all-glass tissue homogenizer. Five grams of weighed tissue are homogen-

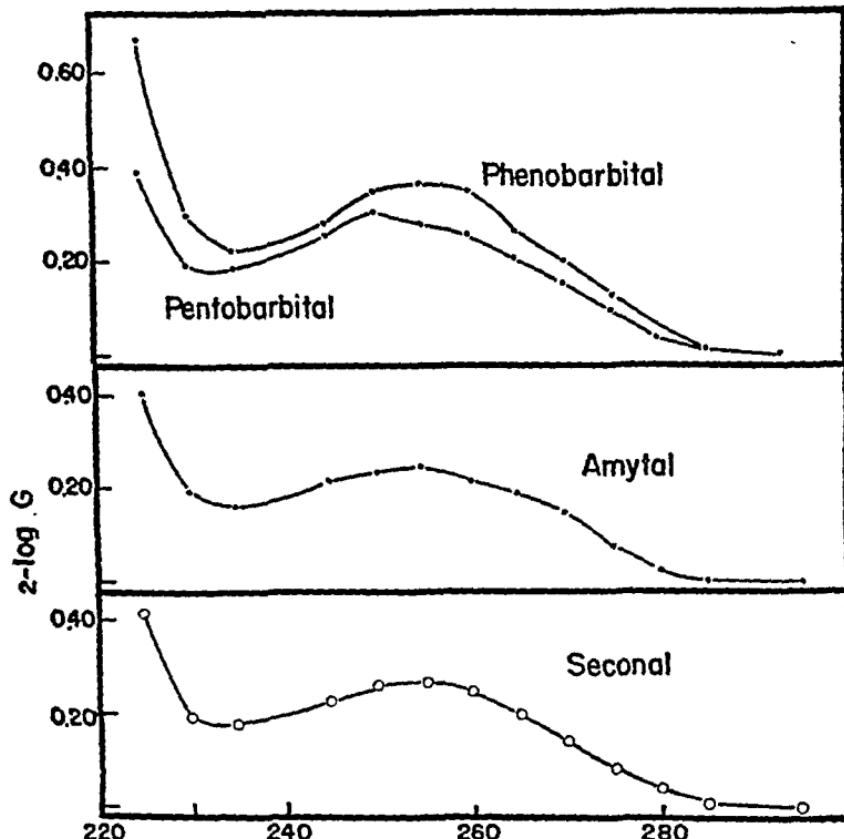


FIG. 1. ABSORPTION SPECTRA OF REPRESENTATIVE BARBITURATES IN CONCENTRATIONS OF 10 MICROGRAMS PER CC. OF 0.5 N SODIUM HYDROXIDE

The ordinate represents optical density (2-log G) and the abscissae wavelength in  $m\mu$ .

ized with 5 cc. of buffer in the blender and with the use of an additional 5 cc. transferred to a separatory funnel containing 50 cc. of extracting solvent. For the accurate analysis of  $\frac{1}{2}$  to 2 grams of tissue, 5 grams are ground up with the buffer in the glass tissue homogenizer, made up to a volume of 25 cc., and an aliquot taken for analysis. Chloroform forms troublesome emulsions with brain homogenates, but if ethylene dichloride is used instead this does not occur. Chloroform is the solvent of choice for all other tissues. The procedure is continued as described in the analysis of blood. The concentration of the

alkali used for the extraction of the solvent is increased to 1 N to eliminate the turbidity that sometimes appeared in the weaker alkali. For the reference blank solution, 1 N sodium hydroxide is used.

*Blood and tissue blanks.* Normal blood and tissue extract will have some absorption at all wave lengths. The same kind and approximately the same intensity of absorption were found with specimens of blood and tissues from rats, mice, rabbits, and humans. In experimental studies, the variation of the blood

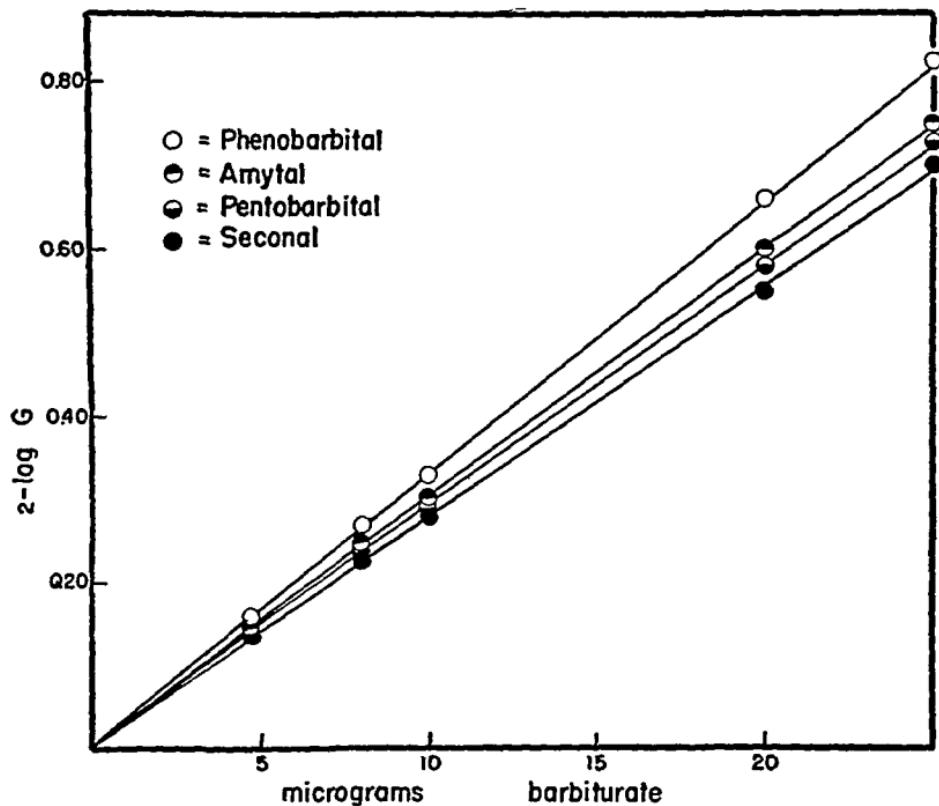


FIG. 2. THE RELATION BETWEEN OPTICAL DENSITY (2-log G) AT 255 M $\mu$  AND BARBITURATE CONCENTRATION

The ordinate represents optical density (2-log G) and abscissae barbiturate concentration in micrograms per cc. of 0.5 N sodium hydroxide.

and tissue blanks, together with those of the reagents, can be controlled by the analysis of a preliminary blood sample taken before the administration of the barbiturate or by the use of tissues from a control animal. For toxicological analysis where no previous barbiturate-free samples are available for controls, known negative blood and tissue, or the average optical density values obtained from a large number of known normal bloods and tissues may be used. The average optical density values for 1 cc. of fresh blood or 1 gram of fresh tissue at 255 m $\mu$  are shown in table I. With putrified tissues, the normal optical density values are increased.

*Recovery of added barbiturates.* Recoveries were carried out by adding known quantities of representative barbiturates (seconal, pentobarbital, amyntal and phenobarbital) to varying amount of blood (0.5 cc. to 5 cc.) and different tissues (0.5 grams to 5 grams). The amount recovered was determined from the difference between the optical densities at 255 m $\mu$  of each sample before and after the addition of the barbiturates. The results are recorded in table II. It can be seen that if the normal blank values are accurately determined, re-

TABLE I  
*The optical densities of extracts of normal tissues at 255 m $\mu$*

TISSUES	NUMBER OF DETERMINATIONS	OPTICAL DENSITIES FOR EXTRACTS OF 1 CC. OR 1 GRAM IN 5 CC. OF 0.5 N NaOH*	STANDARD ERROR OF THE MEAN IN TERMS OF MICROGRAMS OF BARBITURATES
Blood	25	.03 ± .007	1.3
Liver	15	.07 ± .01	1.9
Kidney	12	.06 ± .01	1.7
Brain	15	.04 ± .01	1.7
Muscle	12	.04 ± .01	1.7

\* Includes the standard error of the mean

TABLE II

*Recoveries of representative barbiturates (seconal, amyntal, pentobarbital, phenobarbital) added to blood and tissues*

SAMPLE	NUMBER OF DETERMINATION	QUANTITY OF BARBITURATES ADDED TO SAMPLES OF 0.5 CC. TO 5 CC. OF BLOOD OR 0.5 GRAMS TO 5 GRAMS OF TISSUE	AVERAGE RECOVERY*
			micrograms
Blood	20	20	19 ± .013
Blood	16	40	39 ± .014
Tissues†	25	40	39 ± .020
Tissues and blood	11	80	81 ± .025
Tissues and blood	8	100	97 ± .030

The average recovery was found to be independent of the kind of tissue or barbiturate.

\* Includes the standard error of the mean.

† Combined recoveries from liver, brain, muscle and kidney

coveries of more than 95 per cent are obtainable when barbiturate concentrations as low as 20 micrograms are added to varying amounts of blood and tissue. In toxicological samples, the size of the error that may be introduced by the use of the average normal values in table II is indicated by the standard error of the mean for each tissue. Since a value, to be significant, should be two or three times the standard error of the mean, the procedure is reliable for barbiturate levels as low as 0.4 mgm. per 100 cc. of blood and 1 mgm. per 100 grams of tissue. The error introduced for these low level determinations may be as large as 25

per cent, decreasing with increasing concentrations of barbiturates to less than 10 per cent for levels above 2 mgm. per 100 cc. of blood and 4 mgm. per 100 grams of tissue.

*Sensitivity.* The concentration of barbiturate needed to give a significant optical density value at 255 m $\mu$  was found to be approximately 2 micrograms per cc. of sodium hydroxide (fig. 2) or 10 micrograms in 5 cc. (the volume at the end of the extraction procedure). It was found possible to determine barbiturate concentrations of 0.4 mgm. per 100 cc. of blood and 1.0 mgm. per 100 grams of tissue. The use of a large sample is indicated when the barbiturate concentration is low and the blank is small, for although there is a proportional increase in the blank, more accurate results are obtainable at the higher optical density. A 5 cc. blood sample is taken for barbiturate concentrations between 0.4 to 3 mgm. per 100 cc. Above these levels, 2 cc., 1 cc., and 0.5 cc. are used. A 2 gram to 5 gram sample is taken for barbiturate concentrations of 1 mgm. to 3 mgm. per 100 grams, and 1 gram or less for higher concentrations.

*Specificity.* The presence of a barbiturate in the extract of blood or tissue may be demonstrated by obtaining the optical density of the unknown sample at various wave lengths between 225 m $\mu$  and 305 m $\mu$ , then subtracting the optical densities at the respective wave lengths obtained from a similar quantity of normal blood and tissue. The resulting absorption curve should be identical with those in fig. 1 and have a characteristic maximum at 255 m $\mu$  and a minimum at 235 m $\mu$ .

An additional characteristic of barbiturates is the difference in the absorption spectra in alkaline and in acid solutions. There is a marked shift in absorption maximum from 255 m $\mu$ , in alkali, to a wave length below 220 m $\mu$ , in acid. The amount of change is directly related to the concentration of barbiturate in solution.

Differences in the absorption spectra of the barbiturates are not great (fig. 1). However, they are sufficient to produce a change in the ratios between the optical densities at 235 m $\mu$ , 230 m $\mu$ , and 225 m $\mu$  with that at 255 m $\mu$ . For example, the ratios for phenobarbital are 0.69-0.97-2.06 in contrast to 0.59-0.70-1.55 for seconal. These ratios can be used to differentiate some of the barbiturates.

*Determination of blood and tissue levels in rabbits and humans.* To illustrate the application of the above procedure, rabbits weighing approximately 3 kgm. were injected intravenously or intraperitoneally with each of four barbiturates representing various degrees of duration of action. Blood samples were withdrawn by cardiac puncture at different time intervals. Finally, after killing the rabbits with an air embolism, the distribution of the barbiturates in the tissues was determined. The results of this study are shown in table III. A day-by-day study of the blood level of a patient given phenobarbital is illustrated in figure 3. The analysis of a number of toxicological specimens are listed in table IV.

*DISCUSSION.* Table III shows that with phenobarbital, a long-acting barbiturate, the blood level falls very slowly and remains at a high level for a considerable time. With the short-acting barbiturates the level falls at a more rapid

rate. The disappearance from the blood is related to the duration of action of the barbiturates, i.e., amyta! falls at a slower rate than seconal. The concen-

TABLE III  
*Blood levels and tissue distribution after the injection of anesthetic doses of representative barbiturates in 3 kgm. rabbits*

BARBITURATE	AMOUNT INJ.	TIME AFTER INJ.	PLASMA	WHOLE BLOOD	LIVER	KIDNEY	BRAIN	MUSCLE
			mgm.	min.	mgm. 100 cc.	conc.	conc.	conc.
Phenobarbital	410 I.V.	30	16.2					
		60	14.4					
		130	14.4					
		345	13.8	12.7	20.0	11.5	12.0	9.0
Phenobarbital	420 I.V.	30	17.0	17.0	28.0	13.0	13.5	13.0
Phenobarbital	100 I.P.	10	6.9	6.7	19.8	7.7	3.4	4.8
Amytal	150 I.V.	6	7.8	7.5				
		37	5.3	5.1				
		86	4.1	3.5				
Amytal	100 I.V.	12	5.8					
		42	5.0					
		57	4.0					
Amytal*	100 I.V.	5	7.4	7.0	22	12.4	11.0	5.2
Pentobarbital	100 I.P.	10	4.0					
		37	3.4					
		67	2.5					
		99	2.3	2.0	5.5	4.5	4.6	2.2
Pentobarbital	200 I.P.	15	7.4	7.1	27.0	12.0	8.0	4.0
Pentobarbital	100 I.P.	19	4.2	4.4	17.4	7.8	5.2	3.3
Seconal	100 I.P.	8	5.8					
		19	2.4					
		49	2.4					
		79	2.4					
		104	2.4					
		154	2.0					
		219	1.4	1.4	5.0	2.4	2.0	2.0
Seconal	125 I.P.	5	6.8	6.5	20.0	5.4	3.5	3.8

\* After 57 minutes the animal was reinjected with 100 mgm. of amyta!

tration of barbiturates (table IV) was found to be highest in the liver and lowest in the muscle.

TABLE IV  
*Barbiturate concentrations in cases of non-fatal and fatal poisoning*

CASE	BARBITURATE	STOMACH CON-	BLOOD	LIVER	KIDNEY	BRAIN	CLINICAL HISTORY
			mgm./100 cc.	mgm./100 grams			
1	Seconal	1200	7.5	31.0	24.0	11.0	Found dead
2	Seconal and amytal (Tuinal)	200	3.7				Died 1 hour after admission in coma to hospital
3	Pentobarbital		4.0				Found dead
4	Pentobarbital	1200	4.0	20.0	5.0	4.0	Found dead
5	Amytal		0.8			0.9	Alcoholic: Died soon after injection of 100 mgm. of sodium amytal I.V.
6	Phenobarbital		9.0	9.0			Died 30 minutes after admission in coma to hospital. History of ingestion of paraldehyde and phenobarbital
7	Phenobarbital		9.8				Died 48 hours after admission to hospital in coma
8	Phenobarbital		2.3				Child 3 years old admitted semi-comatose to hospital
9	Phenobarbital		8.0				Comatose upon admission to hospital. 24 hrs. later, semi-comatose
10	Phenobarbital		7.2				Admitted comatose; history ingestion of phenobarbital and paraldehyde
			5.0				24 hours later semi-comatose
			4.4				Six days later, asymptomatic
			2.1				

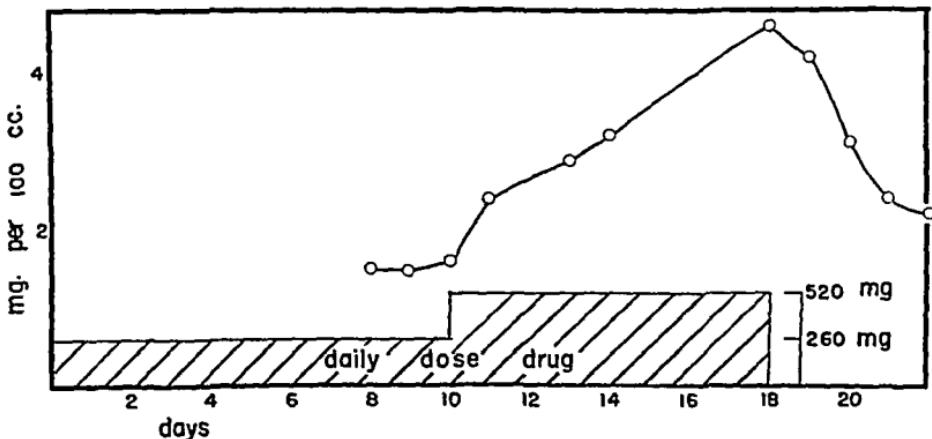


FIG. 3. BLOOD LEVEL OF A PATIENT ON DAILY DOSES OF PHENOBARBITAL

This procedure has been of great value in the clinical, pharmacological, and toxicological studies of the barbiturates by making possible a rapid, simple quan-

titative determination of low barbiturate levels in the blood and tissues. In cases of coma of undetermined origin, the analysis of a blood sample provides a rapid means of qualitatively and quantitatively determining the presence, or absence, of barbiturates. The subsequent treatment can be determined by the barbiturate blood levels found at different time intervals. Further studies are being made to relate barbiturate blood levels with clinical signs and symptoms, as well as the use of this procedure for the toxicological estimation and identification of barbiturates.

#### SUMMARY

1. A simple, rapid, highly specific procedure is described for the determination of barbiturates in blood and tissues based on the characteristic ultraviolet absorption spectra of the malonyl urea ring structure.
2. The method is sensitive to 0.4 mgm. per 100 cc. of blood and 1.0 mgm. per 100 grams of tissue with an error of less than 10 per cent.
3. This procedure is applicable to the clinical, pharmacological, and toxicological investigation of barbiturates in blood and tissue.

*Acknowledgment.* The author is indebted to John Hidalgo and James Spencer for their technical assistance.

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# THE EFFECT ON THE PAIN THRESHOLD OF N-ACETYL p-AMINOPHENOL, A PRODUCT DERIVED IN THE BODY FROM ACETANILIDE<sup>1</sup>

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Recent work has shown that the major part of acetanilide administered orally to man is transformed in the body to N-acetyl p-aminophenol (1, 2). Considerable concentrations of this substance found in plasma suggested that the analgesic activity of acetanilide might be directed, at least in part, through this derived product. Studies which appraise the effect of therapeutic doses of N-acetyl p-aminophenol on the pain threshold are described below.

**METHOD AND MATERIALS.** The technique employed here for the measurement of pain threshold, a modification of the cutaneous heat-radiation method described by Hardy, Wolff and Goodell (3), has been previously described (4). Twelve normal female human subjects, all previously familiarized with the sharply defined pain sensation, were used for the tests.

Duplicate control measurements of the pain threshold were made. These agreed for each subject within 3 per cent. N-acetyl p-aminophenol was administered orally, and observations of the pain threshold level were made at 30 minute intervals for 4½ hours. The pain threshold raising effect was expressed in per cent elevation above the control level.

**RESULTS.** Figure 1 shows the time-effect curves for N-acetyl p-aminophenol after the administration of 0.325 grams of the drug. Twelve experiments were done, so that each point is the average of 12 measurements. The threshold to pain rose within 30 minutes following the administration. The peak of the action was reached in about 2½ hours and the threshold returned to normal in about 4 hours. Plasma levels were not measured in these experiments. However, the time of the maximal analgesic effect and the duration of the effect were consistent with the plasma levels obtained in other subjects after oral administration of the drug (2).

The maximal rise in pain threshold, above the control level, averaged 30 per cent. This rise was maximal for the drug since the administration of 1 gram achieved no further increase in the pain threshold. The height and duration of the threshold raising effect of N-acetyl p-aminophenol were comparable with those obtained by other workers with acetanilide (5).

Placebos were administered to the subjects used in the above experiments and the change in pain threshold was measured. The maximal rises above the control level averaged four per cent.

**DISCUSSION.** The analgesic activity of N-acetyl p-aminophenol in therapeutic

<sup>1</sup> The work in this paper was supported by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

dosage was found to be comparable to that of acetanilide administered in the same dosage. The rapid transformation in man of acetanilide to N-acetyl p-aminophenol suggests that acetanilide acts for the most part through this derived compound. It has been shown that acetophenetidin (p-ethoxy acetanilide) is rapidly changed in the body to N-acetyl p-aminophenol suggesting that acetophenetidin also acts through this derived product (6).

N-Acetyl p-aminophenol, administered orally, is not attended by the formation of methemoglobin even in high dosage, nor at least *in vitro*, does it destroy red cells. It is possible, therefore, that it may have distinct advantages over acetanilide as an analgesic.

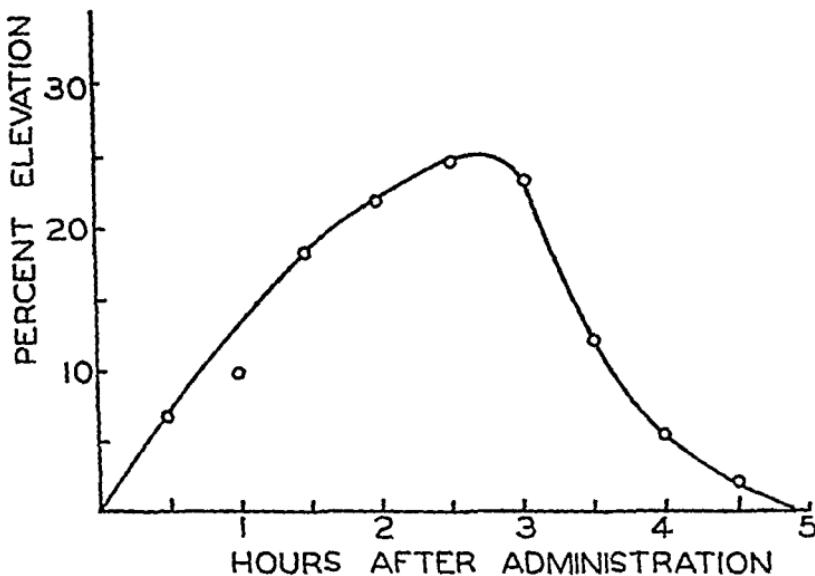


FIG. 1. THE AVERAGE PAIN THRESHOLD ELEVATION FOR 12 SUBJECTS RESULTING FROM THE ORAL ADMINISTRATION OF 0.325 GRAMS OF N-ACETYL p-AMINOPHENOL

#### SUMMARY

The pain threshold elevation of N-acetyl p-aminophenol, a product derived in the body from acetanilide, was assayed by cutaneous heat-radiation. The results indicated it to be an active analgesic. It is suggested that the action of acetanilide is directed through this derived product.

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# ON THE MECHANISM OF THE CONVULSANT ACTION OF STRYCHNINE; THE LACK OF ATROPINE ANTAGONISM

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Strychnine is a powerful convulsant which exerts its major action on the spinal cord. It has been suggested by Nachmansohn (1), on the basis of certain *in vitro* experiments, that strychnine exerts this action by virtue of its ability to inhibit cholinesterase. The pharmacological properties of strychnine, as exhibited in the cat, do not for the most part resemble those of typical anti-cholinesterase drugs. It is unlikely, then, that the mechanism of action of strychnine can be explained on this basis alone. In addition, strychnine is a much less potent anti-cholinesterase *in vitro* than is physostigmine, neostigmine or di-isopropylfluorophosphate (DFP).

The central nervous system effects of anti-cholinesterase compounds are presumed to result from the accumulation of acetylcholine. It is now generally accepted that atropine counteracts the central effects of acetylcholine (2); moreover, it has been shown that atropine annuls or prevents all of the actions of physostigmine and neostigmine in the spinal cord (3, 4) as well as the central excitatory action of DFP in laboratory animals (5) and in humans (6). It should be emphasized that in all these cases relatively small doses of atropine are sufficient to abolish central effects. If cholinesterase inhibition is the mechanism by which strychnine acts upon the spinal cord, then atropine should prove an effective antagonist.

Recently, it has been reported that large doses of atropine exert a protective action against the lethal effects of strychnine in the mouse (7). On the other hand, there are data which suggest that atropine augments the convulsive action of strychnine in the dog (8). The present experiments were designed to investigate the possible antagonism between strychnine and atropine in the central nervous system and to compare the action of strychnine with that of a powerful anti-cholinesterase, DFP.

**METHODS.** Cats were used in all experiments. Under preliminary ether and local anesthesia the trachea was cannulated, screw electrodes were inserted in the calvarium over the frontal areas, and coaxial screw electrodes were inserted in the upper lumbar vertebrae until they touched the spinal cord. When the animals had recovered from general anesthesia they were curarized (Intocostrin, Squibb) to prevent muscle potential artifacts in the electroencephalogram (EEG) and the electrospinogram (ESG); positive pressure artificial respiration at the rate of 20 per minute was instituted when curarization was complete. Records of electrical potentials from the cortex and spinal cord were made with a Grass

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4-channel ink-writing electroencephalograph. In several cases, spinal animals were prepared by sectioning the cord at the level of C-1 under preliminary ether anesthesia.

All injections were made intravenously into a saphenous vein. All solutions were prepared daily in distilled water in suitable concentrations.

**EXPERIMENTAL.** *Effect of atropine.* The effect of atropine on the control EEG and ESG patterns was observed in 4 cats. The injection of relatively small (1-3 mgm.)<sup>3</sup> doses of atropine sulphate produced a shift in the control electrical pattern of the cortex to one in which moderately high voltage waves in the slow frequency range of 6 to 10 per second predominated. Further increments of atropine did not produce any more drastic slowing. In contrast to this, the injection of atropine in amounts ranging from 1 to 40 mgm. did not produce any change in the spontaneous electrical activity of the spinal cord.

*Action of DFP.* Experiments were performed upon 6 cats. The injection of 3 mgm. of DFP (the LD<sub>100</sub> for this sample of the drug) produced, within 2 to 4 minutes, a change in the electrical pattern of both the cortex and the spinal cord. The cortical effect has been described in detail in a previous publication (5); it consists, briefly, in the appearance of low voltage waves in the high frequency range of 24 to 40 per second. In the spinal cord, the onset of DFP activity occurred within 1 to 2 minutes; it produced rhythmic bursts of activity which can be described best as an intermittent tetanus. The frequency of this regular discharge varied between cats but always lay in the range of 26 to 28 per second; this latter frequency appeared to be the maximum which could be attained, for higher doses of DFP did not influence it. These high frequency potentials were of high voltage and persisted for extended periods of time if atropine were not given. Figure 1B shows a typical DFP response.

Similar experiments were carried out on 3 cats whose spinal cords had been sectioned at the level of C-1. In these animals the same cortical effects appeared after DFP as in unoperated preparations, but no spontaneous increase in activity was produced in the spinal cord. No spontaneous increase in voltage or frequency could be detected from the spinal leads although such animals, under these circumstances, had exceedingly active reflexes and responded with short high voltage bursts to jarring or tapping on the vertebral column.

*Atropine-DFP antagonism.* The increase in electrical activity of the cord and cortex produced by DFP was abolished promptly by the injection of 3 mgm. of atropine. This effect of atropine is shown in figure 1. The spinal cord effect was the more easily controlled by atropine; in all instances, the cord activity returned to control levels in 45 to 60 seconds.

It should be mentioned that the longer the interval between DFP administration and injection of atropine, the more difficult it became to reverse the convulsive pattern in the cortex with the latter drug. However, all convulsive activity produced by DFP could be controlled if the doses of atropine were increased to 5 or 8 mgm. One other phenomenon is worthy of note; in an occasional animal the injection of atropine was followed by an immediate short-lived (10 second or less) increase in cortical and spinal activity which then

<sup>3</sup> All doses are expressed in milligrams per kilogram body weight.

fell to control level. This has been observed also in unanesthetized, uncurarized cats undergoing DFP convulsions (9).

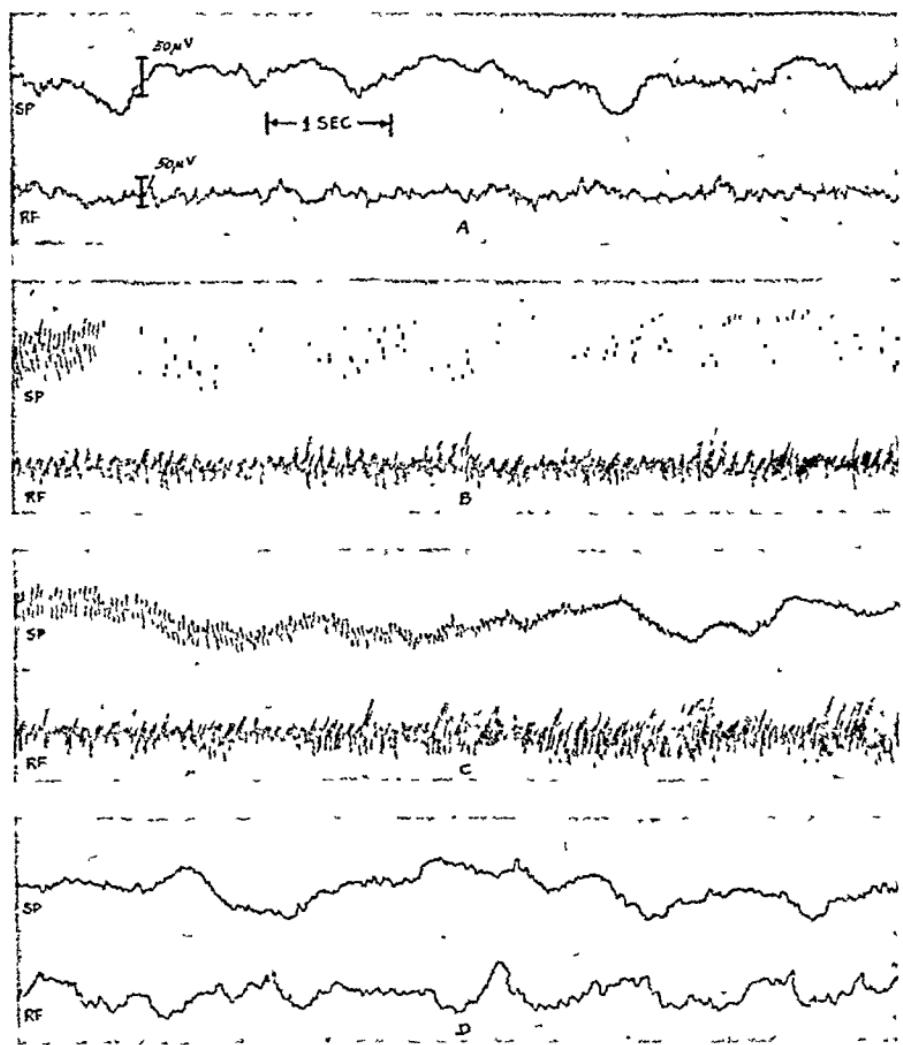


FIG 1 THE ACTION OF DFP AND ATROPINE-DFP ANTAGONISM

Curarized cat, unanesthetized A Control tracing SP—spinal lead, RF—right frontal lead B Tracing obtained 3 minutes after the injection of 3 mgm of DFP C Tracing obtained 50 seconds after the injection of 3 mgm of atropine D Tracing obtained 3 minutes after C

*Effect of strychnine.* The effect of strychnine was observed in 8 curarized cats. In these animals the injection of 0.1 or 0.2 mgm. of strychnine sulphate produced a "spinal convolution" within 30 to 60 seconds. This effect on the spinal cord consisted of the appearance of a long-sustained, continuous series of high voltage potentials with a frequency of 28 per second. This continuous activity lasted for as long as 2 hours in some instances. Despite the tremendous activity

in the cord, the cortex showed very little deviation from control activity in this dose range. A typical strychnine effect appears in figure 2.

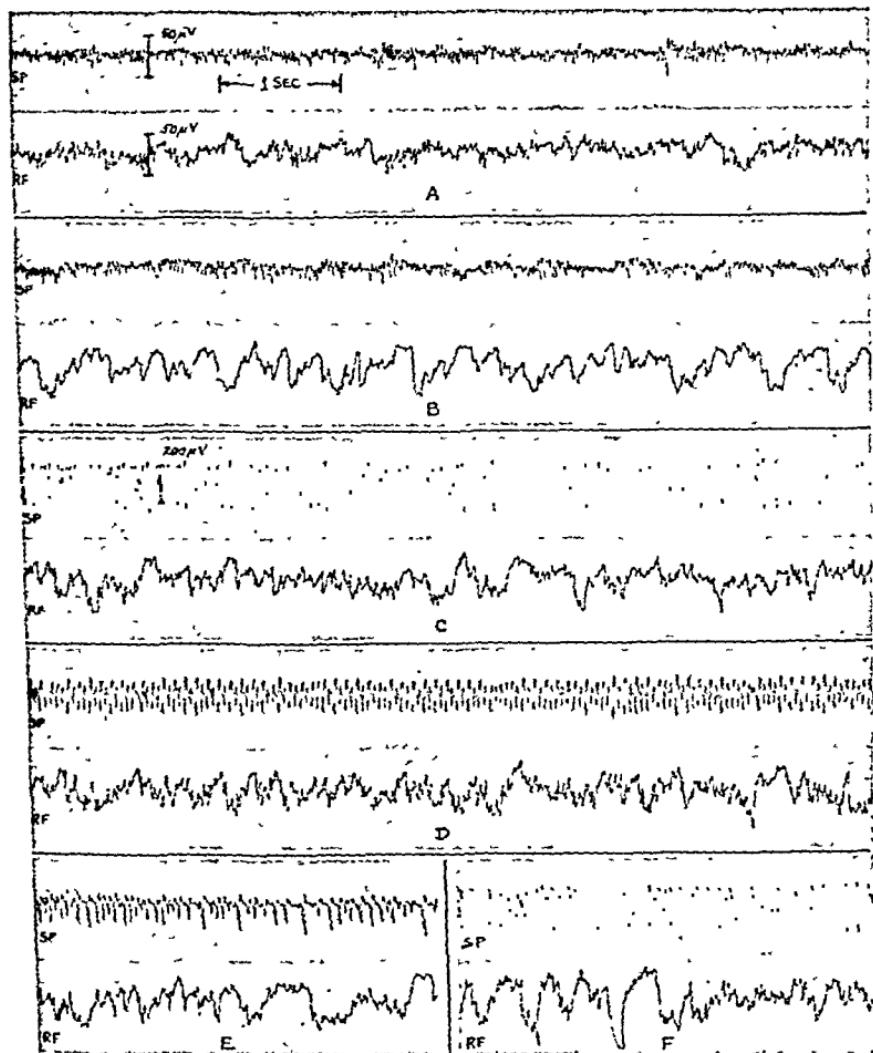


FIG. 2 THE ACTION OF STRYCHNINE AND FAILURE OF ATROPINE ANTAGONISM

Curarized cat, unanesthetized. A. Control tracing SP—spinal lead, RF—right frontal lead. B. Tracing obtained 8 minutes after A. Atropine has been given in divided doses to a total of 20 mgm. C. Tracing obtained 1 minute after the injection of 0.2 mgm. of strychnine. Ten minutes had elapsed since the last atropine injection. D. Tracing obtained 3 minutes after C. E. Tracing obtained 20 minutes after C. F. Tracing 1 hour after C and immediately after tapping on the vertebral column.

Similar experiments were performed on 4 cats with cord transection. In these animals spinal "electrical convulsions" appeared within 45 to 60 seconds. The spinal potentials were more variable in this series; although high voltage waves

of 28 per second frequency appeared, the runs were less well sustained; instead of a long repetitive series, the ESG consisted of relatively short bursts of such

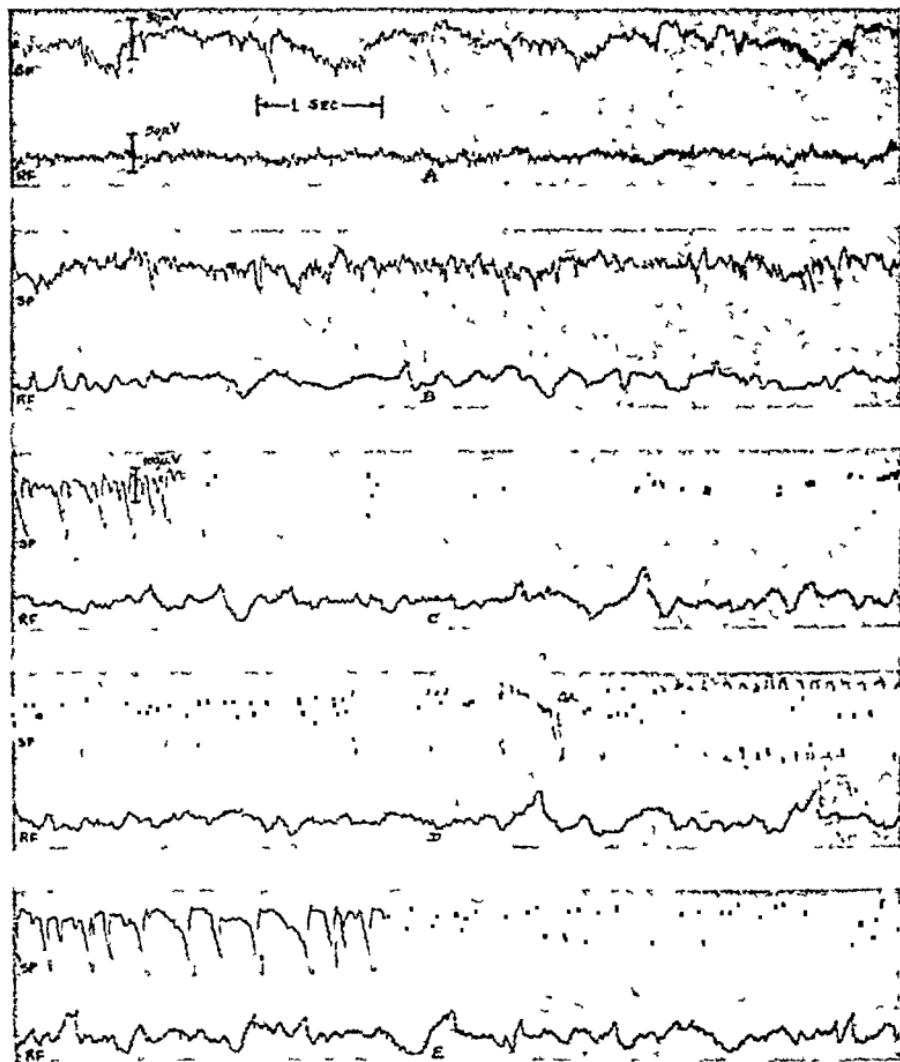


FIG 3 THE ACTION OF STRYCHNINE IN THE SPINAL CAT AND FAILURE OF ATROPINE ANTAGONISM

Spinal cat, curarized, unanesthetized A Control tracing SP—spinal lead, RF—right frontal lead B Tracing obtained 3 minutes after the injection of 20 mgm of atropine in 2 divided doses C Tracing 2 minutes after the injection of 0.2 mgm strychnine D Tracing 1 minute after C E Tracing 3 minutes after C

activity separated by periods of high voltage waves in frequency ranges from 10 to 24 per second. During periods of such random activity, the regular 28 per second pattern could be re-established easily by lightly tapping over the vertebrae or stroking the skin. Figure 3 shows the typical effect of strychnine in the cord-sectioned animal.

*Absence of atropine effect on strychnine action.* In both the spinal and the intact cat the injection of as much as 50 mgm. of atropine did not prevent the onset of electrical convulsions produced by strychnine and could not abolish them once they had appeared. These experiments did not produce any evidence, either electrically or clinically, that atropine exerted any moderating influence on the action of strychnine. This is in direct contrast to the ease with which atropine aborts or prevents spinal and cortical convulsions induced by DFP.

The possibility of atropine-strychnine antagonism was also tested in 4 unanesthetized, uncurarized cats. Each of these cats received 16 mgm. of atropine intravenously 1 hour prior to the injection of 0.2 mgm. of strychnine. All of the animals died in typical tetanic strychnine convulsions; the atropine did not prevent the appearance of symptoms nor did it prevent a fatal outcome. The antidotal effect of atropine in cats poisoned with physostigmine, neostigmine or DFP, on the other hand, is well established.

**DISCUSSION.** The present experiments confirm previous observations that atropine antagonizes effectively the central excitatory actions of DFP. In view of the fact that the great mass of data points to cholinesterase inhibition as the only mode of action of DFP, it is logical to conclude that atropine acts in this case by blocking receptor cells from the action of acetylcholine. Atropine inhibition of DFP effects is even more prompt and efficient in the spinal cord than in the cortex although, unlike its slowing effect on the cortex, atropine exerts no effect on the normal spontaneous activity of the cord.

The observation that DFP does not cause spontaneous spinal convulsions in the spinal animal was unexpected. The additional finding that strychnine convulsions are less well sustained in the spinal animal than in one with an intact neuraxis suggests that pathways from centers above the cord must remain intact, if maximum sustained spinal stimulation is to take place.

The observation that atropine reversal of DFP effects is more difficult to accomplish as the interval between DFP and atropine administration lengthens is not new. Prophylactically, atropine is efficient in smaller doses than it is therapeutically. This phenomenon is probably related to the kinetics of the competition between atropine and acetylcholine for the receptor cell.

The absence of modifying effect of atropine on strychnine action is in direct contrast to that upon DFP. The major portion of strychnine action was on the cord but DFP, in addition to its effect on the cord in the intact cat, also produced intense stimulation of the cortex. Atropine, remarkably efficient as an antagonist to DFP, does not prevent or abolish the action of strychnine even when it is given in massive doses. The facts that strychnine exerts such a specific action on the spinal cord and that its effects cannot be moderated by atropine militate strongly against cholinesterase inhibition as a significant feature of its convulsant action. The pharmacologic facts serve to point out the inherent fallacy in attempting to define drug action on the basis of *in vitro* experimentation alone.

The maximal rate of ESP convulsions after strychnine is worthy of comment. Smyth (10), who recorded electromyograms in frogs poisoned by strychnine, confirmed Buchanan's observation that the so-called strychnine tetanus consists

of a series of very short tetani. The electromyograms showed that these short tetani occurred at a maximum frequency of 10-12 per second; this frequency was attributed to the rate of discharge from the spinal cord. It was impossible in these present experiments on cats to obtain cord potentials of a frequency higher than 28 per second; this was true with both strychnine and DFP. The onset of the 28 per second pattern was synchronous with the appearance of tetanic convulsions in non-curarized animals. This observation indicates that this frequency is the maximal rate with which the synapses involved can react in the upper lumbar region of the cord in cats, assuming that all the cells "fire" synchronously.

#### SUMMARY

1. The electrical manifestations of strychnine action upon the neuraxis of the cat have been studied.
2. Atropine exerts no effect upon these manifestations although it is very effective against those caused by the powerful anti-cholinesterase, DFP.
3. The facts indicate that it is unlikely that strychnine action upon the spinal cord is referable to its anti-cholinesterase properties.
4. Neither strychnine nor DFP spinal convulsions are well sustained unless the neuraxis is intact.
5. The maximal frequency with which synapses react in the upper lumbar region of the cat has been shown to be about 28 per second.

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# NAPHTHOQUINONE ANTIMALARIALS

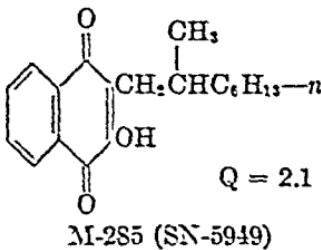
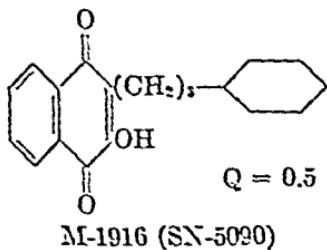
## XVIII. METABOLIC OXIDATION PRODUCTS<sup>1,2,3</sup>

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This investigation is concerned with a series of 2-hydroxy-3-alkyl-1,4-naphthoquinones, exemplified by those coded as M-1916 and M-285, that possess effective



pressive, prophylactic, and curative action against avian malarial infections.<sup>2</sup> In an early clinical trial of M-1916 and M-285 (1), Brodie found that the pigment extracted from red specimens of plasma of patients under study had the same spectral characteristic as the drug administered ( $\lambda_{\max} 490 \text{ m}\mu$  in alkali) but was partially extractable from ether by bicarbonate, whereas the original drug was not. The quinones evidently suffer some form of degradation, and the present investigation was undertaken to discover the nature of the metabolic processes. M-285 is four times as active as M-1916 in the suppression of infections in ducks and chickens but proved completely inactive against *P. vivax* and *P. falciparum* in man, whereas M-1916 exerted a definite, if temporary, therapeutic effect. Both compounds appeared much less effective in man than in birds, probably because of more extensive metabolism. We therefore investigated the metabolic fate of naphthoquinones having various types of side chains in normal and hospitalized nonmalarial subjects in the hope either of finding compounds more resistant to degradation or of isolating a metabolite that might be responsible for the therapeutic action exerted by M-1916.

<sup>1</sup> This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University and in part with the aid of a grant from the Rockefeller Foundation.

<sup>2</sup> Preceding papers: J. Am. Chem. Soc., publication in press.

<sup>3</sup> Paper I surveys the investigations as a whole and includes references to the biological and medical documentation of the compounds.

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TABLE I  
*Administration of naphthoquinones to human subjects*

QUINONE M-, SIDE CHAIN	N. OF CASES	DOSE		SYMPTOMS	PEAK LEVELS OF QUINONE PIGMENT	
		grams	days		Plasma per l.	Urine, per 24 hrs.
1916, -(CH <sub>2</sub> ) <sub>3</sub> C <sub>6</sub> H <sub>11</sub> -Cy.....	13	0.5-2.7	1-14	3/13 GI	12-20	>100*
Acetate.....	1	2.3	4	None	29*	372*
Propionate.....	2	1.8-2.4	1	2/2 M, N	24	90
Hydroq. triacetate.....	1	1.9	2	None	18	34
1971, -(CH <sub>2</sub> ) <sub>4</sub> C <sub>6</sub> H <sub>11</sub> -Cy.....	4	2-2.8	1-3	2/3 N	5-9	206
1956, -(CH <sub>2</sub> ) <sub>5</sub> C <sub>6</sub> H <sub>11</sub> -Cy.....	2	2	2	2/2 GI, N	10-15	10-48
285, -CH <sub>2</sub> CH(CH <sub>3</sub> )C <sub>6</sub> H <sub>11</sub> -n.....	2	1.3-2	1-2	2/2 D	15*	174*
Acetate.....	2	0.7-1	1-3	1/2 D	10	207
1523, -(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub> .....	7	1-4	1-4	2/7 GI	6-21	204
Propionate.....	3	1.3-2.5	1-3	3/3 M	13*	358*
Caprylate.....	2	2	1	None	5-7	
1711, -(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub> .....	1	4.1	1	None	7.7	184
Acetate.....						
1929, -(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> ) <sub>2</sub> .....	7	1-4	1-2	3/7 N, D	6-9	about 100
287, -(CH <sub>2</sub> ) <sub>5</sub> CH(CH <sub>3</sub> ) <sub>2</sub> .....	2	2-3		None	8.7	116
Acetate.....						
273, -C <sub>10</sub> H <sub>21</sub> -n.....	1	2.4	3	None	19*	364*
Acetate.....						
1941, -CH <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub> .....	1	3	2	Slight	1.2	22
Acetate.....						
1933, -(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>2</sub> -CH(CH <sub>3</sub> ) <sub>2</sub> .....	1	2.3	2	1/1 D	1.5	50
Acetate.....						
1538, -CH=CHC <sub>6</sub> H <sub>11</sub> -n.....	1	1	2	None	4.8	14
289, -(CH <sub>2</sub> ) <sub>5</sub> CH=CH <sub>2</sub> .....	1	2	2	1/1 GI	3.2	133
374, -(CH <sub>2</sub> ) <sub>5</sub> -Δ <sup>2</sup> -Cyclohexenyl.....	2	2	1-2	None	6.8	
297, -(CH <sub>2</sub> ) <sub>1</sub> -β-decalyl-trans.....	8	2-3	1-16	None	12-14	8-61
Acetate.....	1	2	1	None	0	
Hydroq. triacetate.....	1	3.5	1	None	0	

TABLE I—Continued

QUINONE M-, SIDE CHAIN	NO. OF CASES	DOSE		SYMPTOMS	PEAK LEVELS OF QUINONE PIGMENT	
		grams	days		Plasma per l.	Urine, per 24 hrs.
2279, -(CH <sub>2</sub> ) <sub>5</sub> -β-decalyl-cis.....	5	2-3	1-11	3/5 M, D	10-14	mgm. low
1952, -(CH <sub>2</sub> ) <sub>5</sub> -C <sub>6</sub> H <sub>5</sub> CH <sub>3</sub> -p Acetate.....	2	2-3	1-2	None	8-10	28
295, -(CH <sub>2</sub> ) <sub>5</sub> -β-tetralyl.....	1	1.8	2	1/1 N	4.6	
266, -Cyclohexyl.....	1	0.6 i.v.		None		

GI = gastrointestinal irritation; D = diarrhea; N = nausea; M = malaise.

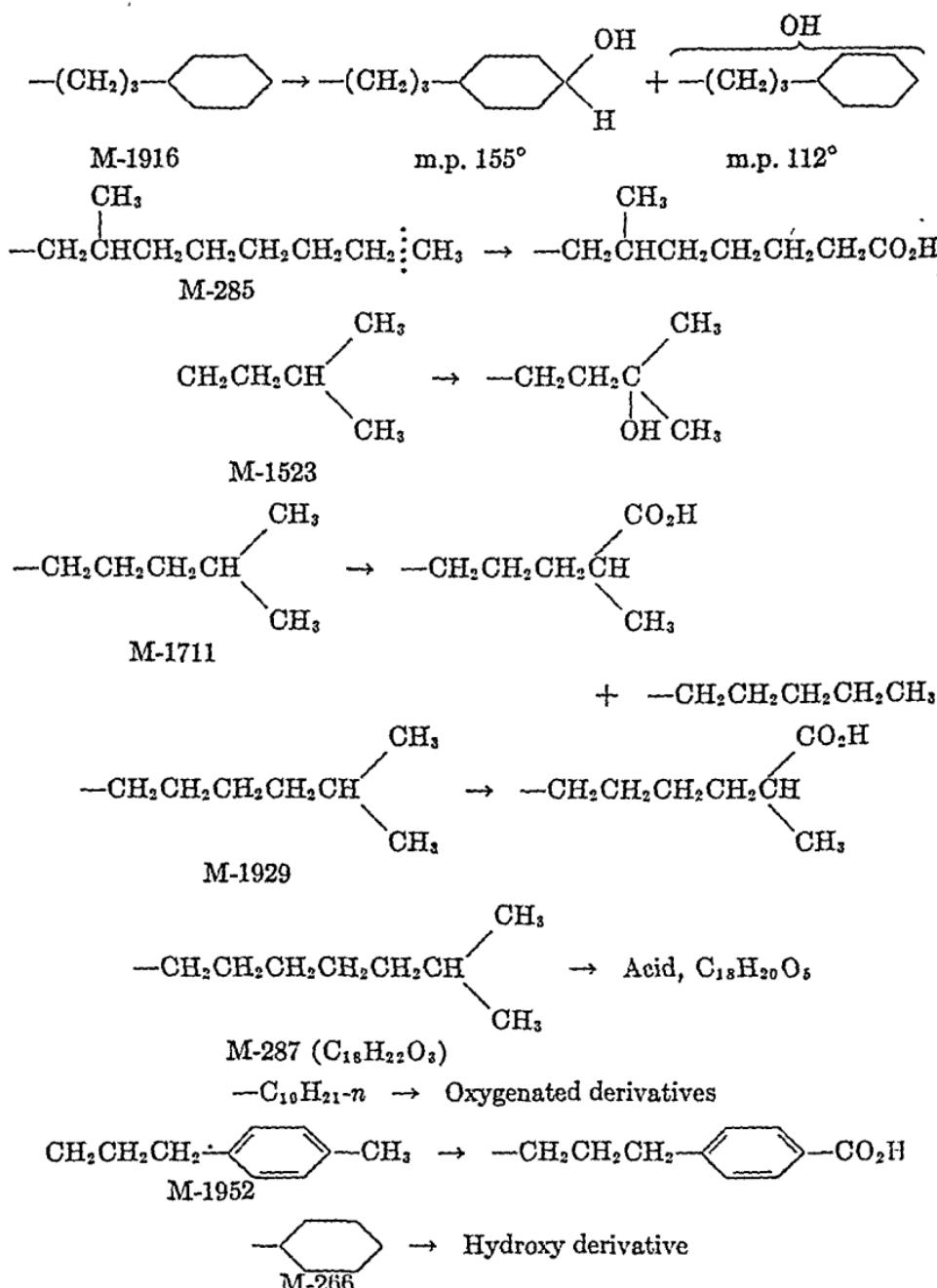
\* The peak values identified by this reference mark were observed in five cases in which drug was ingested by the same individual (L. F. F.)

METHOD. The compounds studied differ only in the nature of the hydrocarbon side chain in the 3-position (table I). The substances were given orally by capsule in the daytime and blood samples (heparinized) usually were drawn the following morning.<sup>7</sup> Plasma analyses were made by early procedures of extraction and colorimetry that are only approximate. Assay by direct polarographic analysis of a mixture of plasma with an equal volume of methanol under nitrogen (M. Fields) proved unsatisfactory because of the presence of variable amounts of reducible substances in normal plasmas. Urines were processed by heating a 24-hr. specimen (rich red) with 50 grams of sodium hydroxide for twenty minutes on the steam bath (deep yellow), cooling, acidifying with 150 cc. of concentrated hydrochloric acid (light yellow), and extracting with ether (two 250-cc. portions). The procedure was applied to a solution of 200 mgm. of hydrolapachol (M-1523) in 1700 cc. of urine and 195 mgm. were recovered. Alkaline hydrolysis in the cold liberates all the pigment but is attended with more troublesome emulsions. When a urine aliquot was not hydrolyzed but treated with quantities of sodium chloride and hydrochloric acid equivalent to the final concentrations of the hydrolysis procedure, the yield of pigment was only two-thirds that obtained by alkaline hydrolysis.

An ethereal extract of hydrolyzed urine, separated after centrifugation or suction filtration to break the emulsion, was washed with small portions of a buffer of pH 6-7 to remove brownish pigment and then separated by extraction with buffers of increasing alkalinity into fractions designated early, middle and late. In some instances plasma samples of sufficient volume (up to 220 cc.) for the isolation of metabolites were obtained by administering the compounds to patients with polycythemia vera before venesection. All substances isolated from plasmas were identical with metabolites found in urines.

<sup>7</sup> No notable differences in drug absorption were observed in trials of: finely ground drug; crystalline sodium salt; drug ground with an equal weight of sodium bicarbonate; ground with glucose; ground with bovine albumin; as low-melting or liquid acetate or higher ester; with enteric coated ox bile tablets; as a liquid (37°) two-phase system (2.5 grams of M-1916, 30 cc. olive oil, 10 cc. 95 per cent alcohol, 2 drops of water). Gastrointestinal disturbances produced by some compounds in some individuals seemed less prevalent when drug was administered with food.

**RESULTS.** The following crystalline metabolites were isolated and identified (indicated by side chain):



The chromophoric group of the hydroxyalkylnaphthoquinones is extremely sensitive to chemical oxidation; for example, the red color of a dilute alkaline

solution is discharged at once when the solution is shaken with ordinary ether that contains peroxide. In the process of metabolic oxidation, however, the first point of attack is at or near the terminal part of the hydrocarbon side chain. When the hydrocarbon group terminates in a cyclohexyl ring, this ring is hydroxylated. In antirespiratory studies the two hydroxylated metabolites of M-1916, which persist in the blood for long periods, have been found to possess one-tenth the antimarial activity of M-1916, and these substances undoubtedly were responsible for the therapeutic effect observed clinically. One compound with a noncyclic (but short) chain, M-1523, is also hydroxylated, but all other open-chain compounds studied are metabolized to carboxylic acids, usually by  $\omega$ -methyl oxidation, but in the case of M-285 by oxidation at the  $\eta$ -position with loss of one carbon atom. Since the carboxylic acids are completely devoid of *in vivo* or *in vitro* antimarial activity, the failure of M-285 in the clinical trial is explained.

The figures given in table I for concentrations of total naphthoquinone pigment represent material that is largely or wholly degraded, for other results (Paper XX) have shown that degradation is complete within two or three hours. The oxygenated and distinctly more hydrophilic metabolites are evidently tolerated in blood at remarkably high levels and they are very persistent. After administration of 2 grams of M-297 per day for three days, the following plasma concentrations were noted (mgm. per cent): day 4, 13.0; day 8, 9.4; day 14, 6.2. When M-1916 acetate was given in olive oil at a dosage of 2.28 grams per day for three days, the levels were: day 4, 27.2; day 7, 27.0; day 14, 7.0. In the latter case the urinary output had dropped by the seventh day from a peak of 0.2-0.4 gram to 0.03 gram per day and the amount excreted from the seventh to the fourteenth day was only about 0.1 gram, and yet during this period the total amount of naphthoquinone circulating in the blood had fallen from an estimated 1.2 grams to 0.3 gram. Since the only quinone pigment recoverable from the feces was pure M-1916, the degraded material evidently undergoes slow oxidation to colorless products. In a trial made to see if continued drug administration would result in the building up of excessive concentrations of degraded material, 1 gram of M-297 was given orally on each of sixteen consecutive days; the basal plasma concentration, determined each morning, soon reached a level of 7-8 mgm. per cent and remained there. The excretion of pigment varies considerably from compound to compound. The continued administration of 2-5 grams per day of M-1916, M-1523 or M-273 results in an output that reaches a peak of 360-370 mgm. per 24 hrs. on the third or fourth day, whereas comparable dosage with M-297 or M-2279 gives an excretion of no more than 60 mgm. per day, and often less.

Table I includes eighteen instances where administration of an acyl derivative of a hydroxynaphthoquinone produced plasma levels of deacetylated drug at least as high as when free drug was given. The hydroquinone triacetate of M-1916 suffered deacetylation in the human organism, but neither the acetate nor the hydroquinone triacetate of M-297 appeared to be assimilated. In four of five instances a propionate produced severe malaise whereas the corresponding acetate, caprylate, or free hydroxy compound gave rise to little or no symptoms.

**EXPERIMENTAL.** *M-1916*—The lower-melting metabolite was first isolated in small amount (m.p. 104–105.5°) from 50 cc. of blood drawn from a normal male on the third day of drug regime (table II) and was then encountered in three plasma samples obtained from polycythemic patients either 4–6 hours after an eight-hour period of drug administration or on the day following three days of dosage (case 27). Still more abundant amounts were obtained from urines. The higher-melting metabolite was encountered less frequently and in smaller amounts, and may arise at a later stage of metabolism. Both substances were isolated from a single plasma sample, both were obtained from the urines of a single individual, and both were obtained after administration of either the free drug or an acyl derivative. No differences were observed in the nature of the mixtures extracted from plasmas and urines.

*112°-Metabolite*—The substance is best crystallized by long standing in a solution in a rather large volume of ethanol highly diluted with water and containing a trace of hydro-

TABLE II  
*Sources of crystalline metabolites from M-1916*

112°-METABOLITE				155°-METABOLITE			
Case	Derivative	Source	Day	Case	Derivative	Source	Day
1	Acetate	L.F.F. plasma	3	7	Clayman* plasma L.F.F. urine Padula urine† Levins urine	Clayman* plasma L.F.F. urine	2 6
5		Ferrar* plasma	1				
7		Clayman* plasma	1				
9		Clayman* plasma	2				
26		L.F.F. urine	4				
		Padula urine†	?				
		Levins urine	3	27	Propionate	Davis* plasma	4

\* Polycythemic.

† Case at Welfare Island.

chloric or acetic acid. The solution very slowly deposits bright-yellow needles, m.p. 111–112.5°. The analyses reported are of two different samples

*Analysis*— $C_{19}H_{22}O_4$ . Calculated, C 72.59, H 7.06

Found, " 72.47, 72.48; H, 7.34, 7.30

Titration at a glass electrode gave the neutralization equivalent 302 (calcd. 314) and the value  $pK^{20}$  per cent acetone = 6.95 (value for M-1916 = 6.67). The ultraviolet absorption spectrum corresponds closely to that of M-1916. The substance is optically inactive. When a sample was acetylated and then treated with pyridine-sulfur trioxide and the reaction mixture distributed between ether and water, 86 per cent of pigment was found in the ether phase; this behavior is indicative of a secondary alcohol (Paper XVI). The sulfate esters of both metabolites behave in very much the same manner and are unlike the esters of tertiary alcohols.

*155°-Metabolite*—The fully purified substance separates slowly from well diluted methanol in the form of long needles. The best sample melted at 152.5–154.5° (microscope).

*Analysis*— $C_{19}H_{22}O_4$ . Calculated, C 72.59, H 7.06

Found, " 72.70, " 7.41

When synthetic 2-hydroxy-3-γ-(4'-hydroxycyclohexylpropyl)-1,4-naphthoquinone (M-2336) became available the following melting points were observed in capillary tubes: M-2336, 155–156°; metabolite, 151.5–153°; mixture, 151.5–154.5°.

*Isolation: Fractionation with Buffers*—Some extracts consisted almost entirely of one or the other metabolite, and fairly pure material was obtained easily by direct crystallization

from ligroin. In case 27 a 100-cc. portion of the plasma was acidified and extracted with ether and the ethereal solution extracted with dilute alkali. The red solution, found by assay to contain 11.5 mgm. of pigment, on acidification deposited crystals of the high-melting metabolite, m.p. 151-152°. Comparable crystalline material was obtained from the total pigment (16.7 mgm.) extracted from a 120-cc. sample of the same plasma with isoamyl alcohol-sodium hydroxide-sodium chloride. The combined mother liquor pigment amounted to only 19.8 per cent of the total pigment, and fractional extractions showed that all of it could be extracted from ether by a buffer of pH 7.66, which is not capable of extracting M-1916. M-1916 is not extracted from even a highly concentrated solution in ether by an 0.067 M phosphate buffer of pH 8.0. Both the 112° and the 155° metabolite can be extracted from a concentrated ethereal solution with fair facility by a buffer of pH 6.8. The extraction constants of the metabolites are both about the same ( $pE = 6.5$ , see Paper XV).

Although initial observations seemed to indicate that a part of the pigment from urines and plasmas could not be extracted by sodium bicarbonate but only by alkali, investigation showed that all the pigment can be extracted from the supposed late fraction with some 15-20 portions of bicarbonate. An empirical procedure for the separation of degraded and undegraded drug by fractional extraction from ether with a buffer of pH 8.6 was applied to the analysis of plasmas drawn at intervals after the intravenous injection in one hour of 500 mgm. of M-1916 in alkaline saline. The estimated amounts of M-1916 present in the total plasma pigment were as follows: immediately after injection, nearly 100 per cent; 1 hour, 52.5 per cent; 3 hours, 11 per cent (4.2 mgm. per cent of pigment).

Another product derived from M-1916 was an early fraction from a third-day urine that remained unextracted by ether after acidification of the total sodium hydroxide extract. This pigment (25 mgm.) was recovered by ether extraction only after the aqueous solution had been saturated with sodium chloride.

M-1911—A fraction of urinary pigment extracted from ether by a buffer of pH 8.25 was sublimed at 140° and 0.05 mm. and crystallized four times from methanol-water; the substance formed yellow needles, m.p. 174.8-176.8°. The composition is not far from that of a monohydroxy derivative possibly contaminated with the product of dehydration formed in the sublimation.

Analysis— $C_{20}H_{22}O_4$ . Calculated, C 73.14, H 7.57

Found, " 74.17, 74.04; H 7.80, 7.64

M-285—The predominant metabolite excreted in abundant amounts in the urine is readily obtained by extracting hydrolyzed or unhydrolyzed urine with ether, extracting the pigment from the ether with several portions of 0.5 M bicarbonate solution, and acidifying the red extract. On standing for a few hours in the cold room the solution deposits a brownish crystallize that is best freed of contaminants by sublimation in vacuum and is thereby obtained as a bright yellow solid. A 13.9-mgm. sample was dissolved in a boiling mixture of 2 cc. of water and 1 cc. of methanol and a pink color was discharged by the addition of one drop of acetic acid. The solution on slow cooling deposited beautiful prismatic needles. When crystallization was effected by adding water to a hot methanol solution to the point of turbidity the substance separated as leaflets. The two forms are interconvertible and have the same melting point characteristics. Four samples were analyzed with the results recorded in table III.

Potentiometric titrations of 5-mgm. samples in 5 per cent acetone solution gave the following results:  $pK^s$  per cent acetone = 5.65, 5.74; neut. equiv., 167, 160 (calcd. for  $C_{18}H_{20}O_5$  with two acidic functions, 158). For comparison, titrations were made with two synthetic acids of comparable molecular weight. 2-Hydroxy-3-(8'-carboxy-n-octyl)-1,4-naphthoquinone:  $pK^{so}$  per cent acetone = 7.13, 7.23; neut. equiv., 169, 161.5 (calcd. 165). 2-Methyl-3-(8'-carboxy-n-octyl)-1,4-naphthoquinone:  $pK^s$  per cent acetone = 4.68;  $pK^{so}$  per cent acetone = 7.35. The titration curves were all similar and those for the two hydroxyquinones showed no break, an indication that the carboxyl and quinonoid hydroxyl groups are of very nearly

the same acidic strength. The spectrum of the metabolite corresponds closely to that of M-285.

The methyl ester was prepared from 21 mgm. of the metabolite, 0.75 cc. of methanol and four drops of concentrated sulfuric acid (overnight, then refluxed). A yellow solid, m.p. 72-79°, that precipitated on the addition of water was sublimed at 70° and 0.01 mm. and crystallized three times from 70-90° ligroin. The ester forms yellow prismatic plates, m.p. 76.5-79.5°, and is extracted from ether by 0.5 M bicarbonate solution.

*Analysis*— $C_{18}H_{22}O_5$ . Calculated, C 69.07, H 6.71

Found, " 68.74, " 6.69

The hydroquinone triacetate prepared from a 40-mgm. sample was obtained initially as an almost colorless glass. A solution of the substance in very dilute methanol slowly deposited colorless warts that melted after recrystallization at 154-160° (microscope).

*Analysis*— $C_{24}H_{22}O_8$ . Calculated, C 64.85, H 6.34

Found, " 64.44, " 6.35

Attempts to decarboxylate the triacetate with copper and quinoline or with copper-barium chromite in diphenyl ether resulted only in the recovery of some of the starting material.

*M-1523*—Hydrolapachol is degraded much less rapidly than any of the other naphthoquinones with a hydrocarbon side chain, and the preponderant constituent of most of the plasma and urine extracts processed was easily isolated and identified by mixed melting

TABLE III  
*M-285 metabolite*

CASE	SOURCE	M.P.	C	H
			"C. per cent	
6	L.F.F. urine	154-174 (microscope)	68.12	6.46
15	H.H. urine	150-174 (microscope)	68.27	6.25
21	Slotnick urine	160-172 (capillary)	68.14	6.03
21	Slotnick urine	163-173 (capillary)	67.90	6.45
		$C_{18}H_{22}O_5$ . Calculated 68.34		6.37

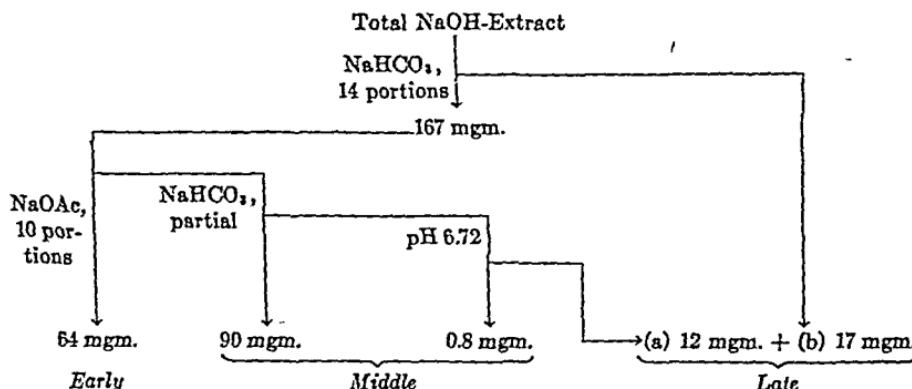
point determination as unchanged M-1523. The first evidence of the presence of a metabolite was the isolation of  $\beta$ -lapachone (2) from an early fraction that had been acidified and allowed to stand in contact with excess hydrochloric acid. The acid-sensitive pigment was then isolated and identified as hydroxyhydrolapachol (2). A comparison of the hydroxy compound with the parent substance showed that the former has an extraction constant about 2 pH units more acidic than the latter. A fairly sharp separation can be accomplished by extraction from ether with sodium acetate, or better with a buffer of pH 6.9; hydroxyhydrolapachol is extracted efficiently whereas very little of the undegraded substance is removed.

In one experiment a polycythemic male was given 4 grams of hydrolapachol caprylate during six and one-half hours and blood was drawn thirteen hours later. The pigment extracted from 215 cc. of plasma was separated by extraction from ether into a sodium acetate fraction containing 1.9 mgm. per cent of pigment (colorimetric) and a sodium hydroxide fraction containing 4.6 mgm. per cent of pigment. Processing of the early fraction afforded a crystalline product melting at 124-126° (microscope) and identified as hydroxyhydrolapachol (m.p. 129-130°) by mixed melting point determination (124-129.5°). The late fraction yielded 3.5 mgm. of hydrolapachol, m.p. 87-90°. The pooled urine of days 2, 3 and 4 was fractionally extracted with a buffer of pH 6.9 and then with alkali with the following results: early fraction, 44.0 mgm. (characterized by conversion to  $\beta$ -lapachone, m.p. and mixed m.p. 153-154°); late fraction, 88.5 mgm. Extraction of the late fraction with petroleum ether and crystallization from dilute methanol afforded 34.7 mgm. of hydrolapachol, m.p.

92.5-93°, mixed m.p. 92.5-94.5°. The mother liquor material was treated with sulfuric acid and residual hydrolapachol removed by extraction with alkali. The neutral residue contained no more than 0.5 per cent of degraded drug. The figures cited indicate that 29 per cent of the plasma pigment is degraded and 33 per cent of that in the urine is degraded.

Analyses of urines of three malarial patients undergoing trial therapy with hydrolapachol propionate indicated that in the first few days of therapy the pigment excreted in the urine was degraded to the extent of 22-33 per cent and that in the period of excretion two or three days after termination of therapy the pigment was 60-80 per cent degraded.

M-1711—In the one case studied the subject was given 4 grams of M-1711 acetate in eight hours. The total pigment from the first 48-hour urine was extracted from ether by alkali and then taken into ether again and fractionally extracted by the process shown in the chart (extracted material down, unextracted to the right); the amounts in each fraction were determined colorimetrically.



*Early Fraction*—The material recovered from the sodium acetate fraction was a dark, partly crystalline solid. Extraction with several portions of 70-90° ligroin gave material that on two recrystallizations from well diluted methanol formed yellow blades softening at 158° and melting at 166° (found C 67.80, H 5.68). A purer sample was obtained by crystallizing the dark ligroin residue from 25 cc. of water; filtration of the hot solution removed considerable tar. The dull yellow needles deposited on cooling were recrystallized from water (20 cc.), alcohol (2 cc.) and a drop of acetic acid. The solution after clarification with Norit deposited short, hair-fine yellow needles that softened at 175° and melted at 186° (microscope).

*Analysis*—C<sub>15</sub>H<sub>14</sub>O<sub>5</sub>. Calculated, C 66.66, H 5.60  
Found, " 67.17, " 5.91

Terminal melting points observed under a polarizing microscope were as follows: synthetic 2-hydroxy-3-(4'-carboxyamyl)-1,4-naphthoquinone (Paper X), 185°; mixture with metabolite, 186°.

*Late Fraction*—The material (b) that had persisted after fourteen extractions with bicarbonate and therefore behaved like undegraded drug gave three crops of silken needles forming voluminous tufts. The first crop on recrystallization from methanol-water afforded 10 mgm. of apparently homogeneous needles, m.p. 102-103°. The melting point is distinctly lower than that of M-1711 (120°) and the analysis corresponds better with that calculated for the next lower homolog.

*Analysis*—C<sub>14</sub>H<sub>12</sub>O<sub>5</sub>. Calculated, C 73.75, H 6.60  
Found, " 73.63, " 7.02

The remainder of the sample was combined with the other crystallizes and the material (13.7 mgm.) was processed with acetylsulfuric acid in pyridine with the idea of removing any unsaturated material (Paper XVI). The mixture was distributed between water and ether

and the fraction in the ether layer was saponified and the red solution (containing 12 mgm. of pigment) acidified. A bright yellow solid separated, m.p. 97-99°, and on crystallization from methanol-water formed silken yellow needles, m.p. 102-102.5°. 2-Hydroxy-3-n-amyl-1,4-naphthoquinone (M-1710) melts at 103-104° (3), and a mixture of this substance and the metabolite in a capillary tube melted at 100-101°, resolidified at once when removed from the bath, and remelted at 99-100.5°. Samples examined under the microscope showed the following terminal melting points: substance isolated, 107°; M-1710, 106.5°; mixture, 105°.

The pigment of the sub-fraction (a) likewise appeared to contain M-1710, for it afforded yellow needles, m.p. 94-96°, that gave no depression when mixed with M-1710 but depressed the melting points of both the *n*-butyl and the *n*-propyl derivatives.

*Other Observations*—The middle fraction afforded no crystalline material. The combined urine of the third and fourth day was extracted as before and the total sodium hydroxide extract acidified and extracted with ether. It was noticed that considerable yellow color was retained in the water layer even after repeated extraction with ether, and therefore the solution was saturated with sodium chloride and again extracted. Ether then indeed removed material giving a characteristic red extract with alkali (27 mgm. pigment). The pigment was recovered by saturation with salt and extraction and obtained on evaporation of the dried ether solution as a sirup that could not be redissolved in ether. It was taken up in about 4 cc. of water and the solution filtered and eventually evaporated to dryness. The sirup was then dissolved in a few drops of water and the solution on standing for several days deposited a large brown crystalline aggregate of a water-soluble metabolite probably with a sugar-like side chain.

*M-1929*—The plasmas of two subjects given M-1929 contained considerable naphthoquinone pigment of a more hydrophilic character than usual. Analyses of the two first-day plasmas by isoamyl alcohol extraction gave the values 2.7 and 5.2 mgm. per cent, but exhaustive extraction with several portions of ether resulted in the recovery of pigment amounting to 7.3 and 20.6 mgm. per cent, respectively. An aqueous alkaline solution of ether-extracted pigment (11.6 mgm. per cent, 3.7 cc.) was saturated by the addition of 1 gram of sodium chloride and shaken with 15 cc. of isoamyl alcohol; the aqueous layer remained red and had a colorimetric equivalent of 3.8 mgm. per cent.

Ether extracts from urine were washed with a small portion of pH 7.1 buffer to remove brown material and extracted with further portions of the same buffer for recovery of metabolite; the greater part of the pigment is readily extracted and hence degraded. In one instance a solid product that slowly separated from an acidified and chilled buffer extract was sublimed at 120° and 0.05 mm. and crystallized twice from methanol-water. The substance melted at 133.5-138.5° (microscope) and was optically inactive.

*Analysis*—C<sub>17</sub>H<sub>18</sub>O<sub>5</sub>. Calculated, C 67.54, H 6.01  
Found, " 67.41, " 6.32

In another case the clarified ethereal solution was extracted with alkali and the alkaline solution was washed with four portions of ligroin to remove froth-forming material. The pigment was then taken into ether and the solution extracted with two 150-cc. portions of pH 7.1 buffer and each extract was acidified, heated to boiling and filtered. The crystallizes that separated on cooling were as follows: 25 mgm. fine, bright yellow needles, m.p. 136-137°; 88 mgm., m.p. 132-134°. The melting point of synthetic 2-hydroxy-3-(5'-carboxy-*n*-hexyl)-1,4-naphthoquinone (Paper X) observed in a capillary tube on the same thermometer was 132-134°, and a mixture of this with the best fraction of the metabolite melted at 132-133.5°, solidified easily, and remelted at 132-134°.

The methyl ester was prepared from the combined crops of metabolite with methanol and hydrogen chloride and a solution of the product in ether was extracted with two portions of pH 7.1 buffer to remove unesterified material. The recovered ester crystallized from slightly diluted methanol as short, flat, yellow needles, m.p. 75-77°.

*Analysis*— $C_{18}H_{20}O_5$ . Calculated, C 68.34, H 6.37  
 Found, " 68.34, " 6.67

A mixture with synthetic ester (m.p. 76.6–77.9°) melted at 76–77°.

*M-287*—Two urines were processed with similar results. Extraction from ether with sodium acetate and then with a pH 6.72 buffer removed 72 per cent of the total pigment and these early fractions afforded an apparently homogeneous substance that crystallized from methanol-water in canary yellow elongated plates melting at 165–170° (microscope).

*Analysis*— $C_{18}H_{20}O_5$ . Calculated, C 68.34, H 6.37  
 Found, " 68.38, " 6.54

The analysis agrees with that of the expected 2-hydroxy-3-(6'-carboxy-n-heptyl)-1,4-naphthoquinone, but the melting point of synthetic acid of this structure (Paper X) is considerably lower (138°). Since the side chain  $-(CH_2)_4CH(CH_3)CO_2H$  contains a center of asymmetry, the metabolite was investigated in a polarimeter and indeed found to be optically active,  $[d]_D^{25} + 24^\circ (\pm 5^\circ)$  in methanol. Attempts to racemize the substance were unsuccessful, and hence the identity remains in doubt.

TABLE IV  
*Metabolism of M-27S ( $C_{20}H_{20}O_5$ ) crystallizes from urinary extracts*

DAY	TOTAL NaHCO <sub>3</sub> FRACTION	M.P. °C.	ANALYSES						Approx. formula	
			Found			Calcd.				
			C per cent	H per cent	O per cent	per cent	per cent			
1 (drug)	79									
2 (drug)	222	90–98	66.96	6.48	26.6	67.09	6.62	$C_{17}H_{18}O_5$		
3 (drug)	297									
4	274									
5	364	112–130	65.83	5.52	28.6					
6	339	135–143	63.64	5.52	30.8	64.14	5.70	$C_{17}H_{18}O_5$		
7	242	138–154	64.36, 64.65	4.82, 5.14	30.5 av.	64.55	5.10	$C_{17}H_{18}O_5$		
8	144	143–146	62.73	5.43	31.8	62.73	5.92	$C_{16}H_{18}O_5$		
9	22									

Both urines afforded the same acid and also, in the late fraction, a substance that crystallized in long yellow needles, m.p. 96–98°. The melting point is 14° lower than that of the undegraded drug and the substance gave a depression when mixed with either the *n*-C<sub>8</sub> or *n*-C<sub>7</sub> derivative.

*M-27S*—The investigation of this compound was conducted in such a way as to characterize the progress of metabolic oxidation; the urine of each successive day was processed in a standard manner to give a crystallizable mixture suitable for analysis. Drug was taken for three days and each 24-hour urine (1200–1800 cc.) was hydrolyzed and extracted with a 200-cc. and a 150-cc. portion of *n*-butanol (the mixture is allowed to stand overnight for clarification of the aqueous layer). The combined extract, with added ether washings, was filtered and extracted with 0.2 N alkali, the extract was washed with ether to remove butanol, acidified, and the pigment taken into ether. The ethereal solution was filtered and the pigment extracted with bicarbonate, determined colorimetrically, recovered with ether, and the residue boiled with successive portions of 70–90° ligroin as long as any yellow color was removed (from time to time the dark tarry residue was dissolved in ether and the solvent evaporated). The combined ligroin extract on standing deposited a large crop of crystalline yellow material and this was recrystallized two or three times from methanol-

water and submitted to analysis (each sample appeared to consist entirely of naphthoquinone derivatives). In the case of the terminal urines the methanol solution could be diluted very extensively without becoming turbid.

Titration of the seventh-day crystallize at the glass electrode indicated the presence of a carboxyl group:  $pK^{10}$  per cent acetone = 5.70, 5.47; neut. equiv., 143, 135 (calcd. for a dibasic acid  $C_{17}H_{18}O_6$ , 158).

The results summarized in table IV show that the composition of the crystallize changes from day to day; the oxygen content rises from 26.6 per cent on the second day to 31.8 per cent on the eighth day. The calculated formulas represent only averages of components of mixtures but they suggest that these mixtures contain  $C_{15}$ - and  $C_{17}$ -diols, triols, acids, hydroxy acids and keto acids. The material excreted on the second day, when drug was still being taken, appears to contain three carbon atoms less than M-273.

**M-1952**—Practically all the pigment extracted from urines and plasmas was found to be extracted by a pH 6.72 buffer, which does not extract M-1952. Extracts obtained with this buffer from urines of days 2, 3 and 4 (two-day drug regime) yielded crystallizes melting in the range 190–211°. One sample for analysis, m.p. 210–211°, corr., was crystallized from methanol-water; another, m.p. 209.4–210.7°, corr., was sublimed and then crystallized.

*Analysis*— $C_{20}H_{16}O_6$ . Calculated, C 71.42, H 4.80

Found, " 71.76, 71.82; H 5.39, 5.15

**M-266**—The total pigment obtained from a urine was extracted repeatedly from ether with an alkali-salt solution of such a concentration as to distribute any M-266 into the ether phase as sodium salt, and the early fraction recovered from the aqueous extracts was further processed by partial extraction from ether with a pH 8.0 buffer. The initial extracts afforded a solid that crystallized slowly from dilute methanol in stout needles, m.p. 196.5–198°. The analysis suggests that the substance is a monohydroxy derivative.

*Analysis*— $C_{16}H_{16}O_4$ . Calculated, C 70.57, H 5.92

Found, " 71.14, " 5.60

*Other Compounds*—The fractional buffer extraction of the urinary pigments derived from the administration of M-1941, M-1933, M-1538, M-289 and M-374 yielded no crystalline products but afforded convincing evidence that these substances suffer very extensive degradation; in each instance the greater part of the pigment could be extracted easily with a buffer 1–1.5 pH units more acidic than one capable of extracting the undegraded naphthoquinone. In the case of the  $\alpha$ -alkenyl derivative M-1538 the inference from buffer fractionation was confirmed by the observation that none of the alkaline extracts showed the purple color characteristic of the starting material.

The remaining compounds listed in table I, M-1956, M-297, M-2279 and M-295, presented difficulties probably associated with the fact that they are of somewhat higher molecular weight than the other substances studied. The excretion in the urine was too low to permit isolation of metabolites, and fractionation with buffers in the alkaline range was found to be unreliable and sometimes misleading.

#### SUMMARY

A study of the metabolism of nineteen 2-hydroxy-3-alkyl-1,4-naphthoquinones in human subjects has established that these substances suffer rapid and extensive degradation consisting in oxidation of the hydrocarbon side chain with the production of metabolites that are distinctly more hydrophilic than the compounds administered. Several of the metabolites were isolated and fully identified; they are secondary alcohols, tertiary alcohols, or carboxylic acids.

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# NAPHTHOQUINONE ANTIMALARIALS

## XIX. ANTIRESPRATORY STUDY OF PROTEIN BINDING<sup>1</sup>

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Wendel (1) has shown that the hydroxyalkylnaphthoquinones are potent inhibitors of the respiration of parasitized red cells of infected ducks and that *in vitro* activities determined manometrically parallel *in vivo* activities. We installed this *in vitro* test method partly for the screening of new compounds<sup>3</sup> and partly in order to expand the study of drug degradation reported in Paper XVIII. The present paper is concerned particularly with standardization of the method and with its use in the investigation of naphthoquinone-protein interactions.

**METHOD.** The procedure of Wendel (1) was adopted with a few minor changes. *P. Lophurae* infection was maintained by blood transfer in 4-6 week old white Pekin ducklings with an amount of inoculum so chosen as to produce a parasitemia of 50-75 per cent on the fourth or fifth day after infection, when blood was drawn for the respiratory studies (reticulocytosis generally was negligible at this time). The red cells were washed and suspended in a duck serum adjusted as follows: removal of carbon dioxide, addition of disodium phosphate to a concentration of 0.025 M, and adjustment of the pH to 7.6 with isotonic alkali. The suspension was then fortified with 10 per cent glucose solution to a glucose content of 100 mgm. per cent. When the parasitemia exceeded 70 per cent the concentration of cells in the suspension was adjusted to a hematocrit of 5 per cent; for infections of 60-70 per cent and 50-60 per cent hematocrit values of 7.5 per cent and 10 per cent, respectively, were maintained. No attention was paid to the relative abundance of the various forms of the schizogonic cycle unless the smear showed an obvious preponderance of segmenters or small rings, when a higher cell content is required. The volume of respiration usually was in the range 250-400 cmm. per hr.

Graded concentrations of the quinone and of the standard M-1916<sup>4</sup> were added to the Warburg vessels and the rate of respiration observed for one hour. A given result is expressed in terms of the IC<sub>50</sub> value (Inhibitory Concentration), the molar concentration required to cause 50 per cent inhibition (1), and the relative potency is given by the ratio of the IC<sub>50</sub> values. This mid-point concentration was always determined by interpolation between points on either side of the 50 per cent line.

Solutions of pure naphthoquinones in alcohol were found to deteriorate appreciably on standing for only a few days, but stock solutions in 0.001-0.005N alkali (containing up to 5 per cent alcohol when required) have shown no sign of deterioration over periods of a few weeks. The solutions in the Warburg flasks may contain as much as 0.5 per cent alcohol by volume, corresponding to 5 per cent of alcohol in the stock solution, but alcohol in this concentration is without influence on respiration. A stock solution is conveniently prepared by dissolving an accurately weighed sample of about 20 mgm. of the quinone in the

<sup>1</sup> See Paper XVIII (This Journal) for acknowledgements to the Committee on Medical Research and the Rockefeller Foundation.

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<sup>3</sup> Papers XXI and XXII, submitted for publication in the Journal of Biological Chemistry.

minimum amount of warm ethanol (up to 5 per cent) in a 500-cc. volumetric flask and adding from 10 to 50 cc. of 0.05*N* sodium hydroxide and diluting to volume with water.

In the typical procedure an alkaline stock solution is diluted to the desired concentration with isotonic saline and a 0.2-cc. portion of the test solution is added to 1.8 cc. of cell suspension in the Warburg flask. However, the sodium salts of some of the compounds are too sparingly soluble to permit the preparation of such nearly isotonic test solutions in concentrations above the IC<sub>50</sub> level. Fortunately, the respiration of cell suspensions is not influenced by changes in the salt content from 0.25 to 1.96 per cent; hence in these special instances the stock solution was diluted with water rather than saline.

TABLE I  
*Change in acidity (from pH 7.6) after one hour of incubation (41°)*

COMPOUND	CONCENTRATION	INHIBITION	FINAL pH	HEMATOCRIT OF CELL SUSPENSION	PARASITEMIA
	mgm. per l.	per cent		per cent	per cent
M-1916	.8	64	7.30	5	77
	.4	57	7.24		
	.2	44	7.30		
M-2293	.3	65	7.31		
	.15	63	7.31		
	.07	55	7.31		
Controls	—	—	7.34		
M-2279	.6	66	7.30	10	55
	.3	51	7.24		
	.15	42	7.23		
Controls	—	—	7.44		
M-2279	.6	75	7.46	5	80
	.3	25	7.49		
	.15	24	7.49		
Plasma extract	6.26	76	7.51		
	3.3	74	7.49		
	1.56	63	7.49		
Plasma extract	.78	42	7.48		
	.39	11.2	7.48		
	.195	6.5	7.51		
Controls	—	—	7.52		

Since the introduction of the usual absorption papers caused the potassium hydroxide to creep from the center well despite the presence of a ring of grease, we abandoned the use of papers and found that then phenolphthalein solution introduced into the flasks invariably remained colorless during agitation for one hour. The rate of CO<sub>2</sub> absorption was satisfactory, and no difference in the rate of respiration of parasitized red cells could be detected in parallel runs with and without absorption papers.

Wendel's recommendation was followed to use 0.025*M* phosphate buffer in the suspending medium to counteract acidosis; higher concentrations maintain the pH more efficiently but depress respiration. Dr. E. G. Ball suggested the use of glycerophosphate, but we found this substance to have as marked a depressing action as phosphate in high concentrations. At the end of a run the pH of the solution in each flask is routinely determined, and if there is any variation greater than 0.05 pH unit in those suspensions that are about 50 per cent inhibited the result is discarded or held in suspicion. The change from the

initial pH of 7.6 is ordinarily slight and the final solutions are about 0.3-4 pH unit more acidic than at the start of the experiment regardless of the concentrations of the cell suspensions, the percentage parasitization, or the degree of inhibition (table I). Our experiences are thus different from those of Wendel, who observed a proportionality between the degree of inhibition and the drop in pH in the course of the run.

In view of the possible deleterious effect on parasite respiration of the citrate used as anticoagulant of the blood drawn from infected ducks for the preparation of the cell suspensions, a comparison was made of the respiration and response to a naphthoquinone of two blood samples drawn at the same time, one into 1/9 volume of 3 per cent buffered citrate and the other into heparin solution (1 mgm. of heparin per 4.5 cc. of blood). No significant differences were observed in the respiration per unit cell concentration either with the fresh samples or after they had stood in contact with the anticoagulant for two hours. The values found for the IC<sub>50</sub> of M-1916 in the fresh and aged pairs of bloods, respectively, were: 7.29, 7.32; 7.26, 7.27 × 10<sup>-7</sup> moles per l.

**Colorimetry**—As a prelude to the investigations a series of comparisons were made to see whether, as expected, equimolar concentrations of the red hydroxyalkynaphthoquinone sodium salts in aqueous alkali gave the same tinctorial value. Colorimetric determination of five typical compounds of widely differing molecular weight, made with a Coleman Universal Spectrophotometer at a wavelength of 490 m $\mu$ , gave the following average values for the ratio Molarity × 10<sup>-5</sup>/Optical Density: M-1916, 38.3; M-1971, 37.7; M-297, 41.4; M-1523, 37.7; M-1929, 38.0 (for structures, see table I of Paper XVIII). Quinones with the following oxygenated side chain also showed equivalent absorption: —(CH<sub>2</sub>)<sub>5</sub>CH(CH<sub>3</sub>)-CO<sub>2</sub>H, 38.4; —(CH<sub>2</sub>)<sub>5</sub>C(OH)(CH<sub>3</sub>)<sub>2</sub>, 37.4; —(CH<sub>2</sub>)<sub>5</sub>C(OH)(CH<sub>3</sub>)<sub>2</sub>, 38.6; —CH<sub>2</sub>CH=C(CH<sub>3</sub>)-CH<sub>2</sub>OH, 38.3. However, as will be shown below, solutions of the sodium salts in isoamyl alcohol do not all show the same molecular equivalence in tinctorial power.

The analytical method, however, was recognized as subject to a serious error due to the presence in the isoamyl alcohol extracts of variable amounts of yellow carotenoid pigment that absorb considerable light in the region of optimum absorption (490 m $\mu$ ) of the red naphthoquinone sodium salts. A simple corrective measure is to acidify the red isoamyl alcohol extract and make a second reading of the color density. The yellow acidic form of the hydroxynaphthoquinone has only about 1.3 per cent the absorptive power of the red salt and the absorption is distinctly less than that of the yellow plasma pigment for which correction is to be made. In the working range of concentration where the red salt is accurately determinable the small color density of the yellow form is proportional to the concentration. Thus the true concentration of naphthoquinone pigment can be determined from the difference in color density before and after acidification. The determination can be done either with the use of a calibration curve showing the difference in absorption by the red and yellow forms or by applying a factor (k) relating the difference in optical density of the red and yellow forms in an isoamyl alcohol extract to the molar concentration in an aqueous (or plasma) solution analyzed.

**Plasma Analysis: Isoamyl Alcohol Method**—Richardson (2) in one early procedure extracted the plasma sample with ether and extracted the pigment from the ethereal solution with alkali. Brodie (2) achieved a concentration of the color by extracting the red sodium salt from the alkaline solution into a small volume of isoamyl alcohol. The research group at the Massachusetts General Hospital (2) then found that when the original plasma sample is shaken with isoamyl alcohol and sodium hydroxide-sodium chloride the red sodium salt is extracted efficiently into the organic phase and can be determined directly after clarification of the solution with a few drops of ethanol. We investigated this method further and established in trials with solutions of known amounts of pure drugs in plasma that no large excess of sodium hydroxide-sodium chloride is required to insure complete extraction. With the use of a standard amount of ethanol for clarification, the color density for the extract is proportional to the molar concentration of drug in the aqueous (or plasma) solution analyzed.

Molar concn. in aqueous solution =  $k$  (Density<sub>red</sub> - Density<sub>yellow</sub>). The factor can be determined by applying the standard extraction procedure given below to three or four dilutions of an aqueous alkaline stock solution and determining the optical densities before and after acidification of the extract. Values found for the factor  $k$  on this basis are as follows:

Compound	$k \times 10^{-4}$
M-1916	87.3
M-2309	94.8
M-266	94.7
M-2301	111.7
M-2350	109.8

No explanation can be advanced for the fact that the substances do not show molecular equivalence in color density in isoamyl alcohol as they do in aqueous alkali.

Since the yellow form of the naphthoquinone absorbs so very little light at 490 m $\mu$  in comparison to the carotenoid pigments, the amount of natural yellow pigment in the plasma can be expressed with fair approximation as the milligram equivalents of the naphthoquinone in question corresponding to the total color density and the yellow solution. In some instances plasma pigments have been encountered amounting to as much as 2.5 mgm. per cent expressed as M-1916. An alternate method of correction tried was to discharge the red color of the isoamyl alcohol extract by shaking it with aqueous hydrosulfite. The reducing agent does not alter the color density due to native pigments in extracts of control plasma, but we were unable to find a technique for preventing air oxidation during the process of clarifying the solution. It was later observed that the red color of an isoamyl alcohol extract can be discharged rapidly and permanently by the addition of a drop of sodium hypochlorite solution, but the application to analysis has not been explored.

*Procedure for Plasma Analysis*—A 3.0-cc. portion of plasma is shaken gently for ten minutes with 5.0 cc. of isoamyl alcohol and 3 cc. of 1 N sodium hydroxide in half-saturated sodium chloride solution; after centrifugation to break the emulsion, 3.0 cc. of the extract is removed and diluted with 1.0 cc. of absolute ethanol and the color density determined. Two drops of glacial acetic acid are added to discharge the red color, the color density is determined, and the molar concentration of drug in the original plasma is obtained by applying an appropriate factor.

In a test of the procedure 10.0 cc. of an alkaline stock solution containing 5.36 mgm. per cent of M-2279 was acidified and extracted with ether, the solvent was evaporated, and the residue shaken with 10.0 cc. of human plasma at 41° for one and one-half hours. The resulting clear red solution was analyzed with the following results: M-2279 calcd., 5.16 mgm. per cent; found: 4.84 mgm. per cent (94 per cent recovery); plasma pigments: 1.5 mgm. per cent as M-2279.

*Preparation of Plasma Extracts for Respiratory Study*—Although isoamyl alcohol extracts naphthoquinones (other than carboxylic acid derivatives) very efficiently from alkalinized plasma, no satisfactory way has been found for recovering the pigment from the extract in a form suitable for the Warburg determinations. Considerable experience has been accumulated on the extraction of pigments from acidified plasma with ether in the course of the isolation studies, and careful trials of ether extraction have been made with plasmas containing known amounts of pure drug. The general conclusion reached is that the method is unreliable. The pigments present in plasma following the administration of a drug that suffers extensive degradation to more hydrophilic products can be extracted easily, and at least fairly completely, by ether; the degraded pigments are also readily extractable from ether by alkali. Some pure, undegraded drugs are also rather readily extractable, particularly those of low molecular weight. However, compounds of the nature of the isomers M-297 and M-2279 (mol. wt. 352.5) are not extracted completely, or even nearly so, by a practical number of ether extractions. Furthermore, these and other highly hydrophobic compounds cannot be extracted satisfactorily from ether with alkali.

Satisfactory results were obtained, however, by precipitating plasma proteins with four

volumes of absolute alcohol and washing the precipitate by centrifugation with two or three successive portions of alcohol until the pigment was all removed. The extract is evaporated carefully to complete dryness (see below) in vacuum at 40-50° and the residue is taken up in a known volume of isotonic saline containing 0.002 mole per l. of sodium hydroxide. The resulting pink or red solution usually is too cloudy for direct colorimetric determination of the pigment and hence a portion is submitted to analysis by extraction with isoamyl alcohol after addition of an equal volume of 1*N* sodium hydroxide in half-saturated saline. Other portions can then be used directly in determinations of the antirespiratory potency. In a test comparison a plasma containing degraded M-2279 was analyzed directly and after the alcohol precipitation process just described. The results indicate satisfactory agreement:

	Pigment found (as mgm. per cent of M-2279)	
	<i>Naphthoquinone</i>	<i>Plasma Pigment</i>
Direct isoamyl alcohol analysis.....	5.10	1.40
Alcohol precipitation.....	4.87	1.91

A trial was made to see if the antirespiratory activity of a pure drug would remain unaltered in the process of solution in normal human plasma and recovery by alcohol precipitation. Surprisingly, increased activity was observed in several instances and yet extraction of the untreated plasma by the same process gave no antirespiratory material. Even aqueous solutions of pure drug when processed in the same way gave extracts of increased potency. Then it was found that the trouble lay in the failure to evaporate the extract to complete dryness. When this was done the activity of the extracted material was the same as that of the pure drug.

**RESULTS. Protein Binding**--The difficulty encountered in the ether extraction of naphthoquinone pigments from plasmas following oral administration prompted the following exploratory study of the protein complexes that seem to be only partly split by acids but from which the drug is easily freed by brief treatment with cold alkali in the presence of isoamyl alcohol. In one trial a plasma from a polycythemic patient given M-1971 was dialyzed against running tap water at 12-15° in a cellophane bag for sixty hours and then extracted and analyzed; the analysis was the same as that of the undialyzed plasma. A test with the pure drug showed that the substance diffuses completely on dialysis from alkali against tap water. In another trial pure M-1971 was agitated with normal plasma in an atmosphere of 5 per cent carbon dioxide and then centrifuged. A rich red solution was obtained showing on analysis the very high level of 116 mgm. per cent of drug. Dialysis of this drug-rich plasma for sixty hours was attended with no diffusion.

An investigation was then made of the interaction of M-1971 with fractions of human plasma proteins kindly supplied by Dr. E. J. Cohn (3). Each fraction was dissolved in phosphate buffer at about pH 7 in concentrations roughly comparable to those at which the fractions occur in normal human plasma. Excess solid drug was added, the suspensions were shaken at 37° for twenty hours, and after filtration the pH was determined and an aliquot was analyzed for naphthoquinone pigment by the isoamyl alcohol method. The record of the results in table II indicates both the actual naphthoquinone concentrations and the solubilities expressed as the amount of the pigment taken up per milligram of protein. Fraction V, consisting of almost pure albumin, has decidedly greater dissolving power than any of the other fractions; it dissolves 75 per cent of the total drug taken up by the five separate fractions, but only 35 per cent of that dissolved by

normal human plasma. The experiment with crystalline human serum albumin shows that the high solvent power of fraction V is indeed due to albumin itself and not to small amounts of other proteins.

Three entries in the table refer to experiments made by dissolving fraction V or crystalline albumin in distilled water rather than in a buffer. The nearly isoelectric solutions are about two pH units more acidic than the others and the amount of drug dissolved is distinctly less. These solutions, of pH 4.6-4.9, are red, like M-1971 anion, and yet M-1971 has an acidity constant estimated as pK 5.5.<sup>4</sup> At the suggestion of Dr. E. G. Ball, a colorimetric determination of

TABLE II  
*Solubility of M-1971 in protein solutions*

PROTEIN	PROTEIN CONCEN.	pH	NAPHTHOQUINONE DISSOLVED		DURATION OF SHAKING	LOSS OF PIGMENT ON DIALYSIS
			Concn.	Per mgm. of protein		
None	0	7.95	0.38	γ	15	
Fraction I (fibrinogen)	0.45	6.8	2.6	5	20	100 (60 hrs.)
Fraction II (globulins)	1.0	6.3	0.19	0.19	15	
	1.0	7.15	0	0	20	
	1.0	7.95	1.35	1.3	15	
Fraction II + III	1.6	7.05	11.3	7	20	49
Fraction IV (lipoproteins)	1.0	6.95	11.6	11	20	63
Fraction V (albumin)	3.0	6.9	69.4	23	20	5
	(3.0)	4.6*	(22.9)	(7.6)	15	0
	(6.0)	4.6*	(36.5)	(6.1)	3	
Cryst. human albumin	3.0	6.9	55.3	18	15	
	(3.0)	4.95*	(21.3)	(7)	15	
Human plasma	6	196	33		17	35
Monkey plasma	?	142			17	40
Duck plasma	?	122			17	6.8

\* Solution not buffered.

the fraction V-naphthoquinone complex was made by adding portions of dilute hydrochloric acid to the isoelectric albumin solution until a point was reached where the color of the pigment matched the color observed on looking through two absorption cells, one behind the other, of which one contained an alkaline solution of M-1971 and the second an acidic solution of M-1971 in 50 per cent acetone, each at half the concentration of the albumin solution. The pH at this point of color equality was found to be 3.9, and hence this is the apparent pK of the naphthoquinone prosthetic group in the complex. The substance is thus 1.6 pK units more acidic (apparent acidity) when bound to the protein than when in the free state. The absorption spectrum of the protein-bound

<sup>4</sup> From the data of Paper I and the following colorimetric determinations of pK in 50 per cent acetone: M-1971, 6.8; lapachol and hydrolapachol, 6.2.

anion does not differ appreciably from that of the free anion. The albumin solution that had been acidified to the point of color equivalence in the pK determination deposited no solid on standing for a time. However, when it was acidified to pH 3.0 the red color disappeared and M-1971 was slowly deposited in the form of fine yellow crystals. When this suspension was dialyzed against distilled water the mineral acid apparently was removed, for the quinone redissolved to produce a red solution.

Experiments with 2,3-dimethylnaphthoquinone and with 2-methoxy-3- $\gamma$ -cyclohexylpropyl-1,4-naphthoquinone failed to reveal any comparable tendency to form stable complexes with albumin. The methoxy compound simulates solubility by forming fairly stable suspensions, but these are turbid like chyle, whereas typical hydroxalkynaphthoquinone-protein solutions are optically clear except for the Tyndall effect of the protein.

The dialysis experiments recorded in the last column of table II show that the naphthoquinone drug is held more firmly by albumin than by the other fractions and diffuses through the membrane into tap water to only a minor extent. An experiment was made to see if M-1971 would diffuse through a membrane from one albumin solution to another, but after shaking at room temperature for thirty-six hours the concentrations of the original solution and that surrounding the membrane bag were 50.0 and 0.86 mgm. per cent respectively.

The last three entries in table II represent a comparative experiment made to see if any differences could be detected in the interaction of drug with human, monkey, and duck plasma that might be related to differences noted in both the antimalarial and antirespiratory activities of the naphthoquinone drugs. The results of both the solubility and the dialysis experiments suggest that the drug-binding power of the three plasmas are in this order: human > monkey > duck. Thus a greater firmness of binding to plasma proteins may be one factor accounting for the greater antimalarial effectiveness of substances like M-1971 in ducks than in humans.

Two observations have been made concerning the distribution of naphthoquinones between proteinoid fluids of the human body. In the course of one of the studies of drug metabolism, Dr. A. M. Seligman had an opportunity to observe a possible diffusion of drug into the cerebrospinal fluid. A young male epileptic had been given 2 grams of M-297 per day for three days (case 44), and on the eighth day the plasma levels observed before and after withdrawal of spinal fluid for a pneumoencephalogram were 9.4 and 8.4 mgm. per cent, respectively. The spinal fluid showed no trace of the vivid pink coloration of the plasma samples and hence it is concluded that no drug had diffused into this fluid. Davis (4-7) observed that sulfa drugs diffuse into the cerebrospinal fluid only to the extent to which they are present in plasma in the free condition and not bound to protein, particularly to albumin (8).

The second observation is that little if any naphthoquinone diffuses from the plasma into the red blood cells. A 13.0-cc. sample of heparinized human blood had a hematocrit value of 46 per cent red cells and hence contained 6.96 cc. of plasma; 5.0 cc. of the plasma was shaken with M-1971 at 37° for forty-five min-

utes in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide, and analysis of an aliquot indicated a drug concentration of 85.8 mgm. per cent. A 2.5-cc. portion of this drug plasma was added to the red cells and residual plasma and the suspension was agitated for forty-five minutes under the same conditions of atmosphere and temperature as before. Plasma and red cells were then separated and the plasma was analyzed. The calculated concentration before equilibration with the red cells is 48.2 mgm. per cent; the concentration found after equilibration was 50.4 mgm. per cent, and hence there is no indication that any drug was taken up by the erythrocytes. A similar experiment with the blood of monkeys infected with *P. knowlesi* was likewise negative.

*Protein Antagonism to Drug Action*—The first observation of an antagonistic effect of plasma proteins was made in the course of the studies of the succinate oxidase system (July, 1944, Paper XXI). A trial was made to see whether the determination of antirespiratory activity could be made directly with a sample of plasma containing naphthoquinone rather than with an extract. Neither the plasma pigment nor the pure naphthoquinone exhibited any succinate oxidase activity in the presence of human plasma at the concentrations effective in the absence of added protein. Exploratory studies of the magnitude of the protein effect indicated that human and monkey plasma are about comparable in antagonistic power, whereas duck plasma is only about one-sixth as effective. The albumin fraction V was found distinctly more powerful than any of the other fractions of human plasma or than hemoglobin. Wendel (1) soon found that proteins likewise depress the inhibitory action of the naphthoquinones on the respiration of parasitized erythrocytes and he has since made extensive studies of the general phenomenon and of the relative susceptibilities of different members of the series. The observations to be reported here pertain largely to compounds not included in Wendel's experiments.

Two comparisons were made of the  $IC_{50}$  values found for M-1916 with suspensions of parasitized duck erythrocytes in normal duck serum and in normal chicken serum, respectively, with the following results:

$$IC_{50} \text{ (duck)} / IC_{50} \text{ (chicken)} = \begin{cases} (a) 26.2/120 = 0.22^5 \\ (b) 9.8/110 = .09 \end{cases}$$

From this it appears that the antirespiratory activity of M-1916 is only  $\frac{1}{10}$  to  $\frac{1}{5}$  as great in chicken as in duck serum. The difference in protein antagonism may account for the marked disparity in the dosages of naphthoquinones required to produce a given response in infected ducks and chickens indicated in table III. The entries in Column B were calculated by the method described in Paper II from the results of assays conducted by Coatney against *P. gallinaceum* in chickens (2). Standardization assays gave the following  $ED_{50}$  values for quinine base administered in two doses per day: (20), 16.6, 17.0, 16.6, 17.1, 17.0, 20.0;

<sup>5</sup> In this run the control flasks containing chicken serum respired 32 per cent faster than those made up with duck serum; in the other run the respiration differed by only 8 per cent.

av. = 17.4 mgm./kgm./dose. The average dose of quinine required to produce a 95 per cent reduction in parasitemia is equivalent to 58.8 mgm./kgm./day of quinine bisulfate, which is very close to Richardson's average value for assays against *P. lophurae* in ducks (2):  $ED_{50}Q = 17.35 \times 3 = 52.05$  mgm./kgm./day of quinine bisulfate. The order of relative activity of the eight naphthoquinones listed in table III is much the same in the two sets of assays, but, whereas nearly the same amount of quinine is required to produce a comparable response against the two species of plasmodia in the two test birds, the average effective dose of naphthoquinone per kgm. per day in the duck test is only 16 per cent that required in the chick test. The difference cannot be due to a difference in efficiency of absorption from the gut, for naphthoquinones give rise to

TABLE III  
Comparison of assays in ducks and in chickens  
 $ED_{50}$

(Effective dose required to produce a 95% reduction in parasitemia, corrected to the standard:  $3 \times 17.35$  mgm./kgm./day of quinine bisulfate)

COMPOUND, M.	A <i>P. lophurae</i> (DUCKS)	B <i>P. gallinaceum</i> (CHICKENS)	A/B
2279	3.5	27.2	0.13
285	4.4	22.1	.20
380	6.7	77	.09
297	8.6	31.6	.27
1971	11.4	112	10
1929	16.1	171	.09
1916	21.4	158	.13
1523	68	(221)	.30
Average ...			0.16

distinctly higher blood levels in chickens than in ducks, and hence may be attributed to the demonstrated greater antagonistic action of chicken plasma proteins.

For the purposes of our specific project in chemotherapy it was particularly desirable to obtain data on the relative activities in duck and human plasma of members of the naphthoquinone series that in other respects have appeared promising, for with this information it should be possible to judge which of a group of compounds of high antimalarial activity against *P. lophurae* in ducks are the most likely to exhibit high activity in man. Human plasma proteins have such a powerful antagonistic effect that high concentrations of drug have to be used in the determination of  $IC_{50}$ . Since it was desirable not to exclude sparingly soluble compounds from the study, and because we planned to investigate the protein susceptibility of plasma extracts that would be available in only very small amounts, we adopted a test procedure in which the  $IC_{50}$  of a compound in duck serum is compared with that in a medium consisting of duck serum containing a fixed proportion (16 per cent) of human serum. In the more recent

experiments the  $IC_{50}$  for M-1916 is determined at the same time in the two media and the results expressed as the susceptibility to the human protein relative to that of M-1916. Table IV gives the protocol of a typical experiment, and table

TABLE IV  
*Procedure for determining relative human-protein antagonism*

CONTROL	3 FLASKS	3 FLASKS	3 FLASKS	3 FLASKS
1.5 cc. parasite suspension in duck serum				
0.2 cc. saline	0.2 cc. of graded concentrations of M-1916	0.2 cc. of graded concentrations of subject drug	0.2 cc. of graded concentrations of M-1916	0.2 cc. of graded concentrations of subject drug
0.3 cc. adjusted duck serum			0.3 cc. adjusted human serum	
	duck IC <sub>50</sub> M-1916 (a)	duck IC <sub>50</sub> Subj. drug (b)	human IC <sub>50</sub> M-1916 (c)	human IC <sub>50</sub> Subj. drug (d)

$$\text{Calculation: Human-protein antagonism} = \frac{d/b}{c/a}.$$

TABLE V

*Relative susceptibility to human-protein antagonism compared with that to duck-protein*

M- NO.	SIDE CHAIN	$\frac{\text{human}}{\text{IC}_{50}} / \frac{\text{duck}}{\text{IC}_{50}}$		HUMAN-PROTEIN ANTAGONISM B/A
		A, for M-1916	B, for subj. drug	
2293	-trans-4-Cyclohexylcyclohexyl	5.4	1.08	0.2
2289	-CH <sub>2</sub> CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> Cl-p	8.1	2.0	.25
2309	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> OC <sub>6</sub> H <sub>5</sub> -p	6.1	26.4	4.3
		10.5	47	4.5
		8.0	27.4	3.4
2243	-( <ch<sub>2)<sub>4</sub>C(OH)(C<sub>6</sub>H<sub>5</sub>-n),</ch<sub>	7.6	15.5	2.0
2350	-( <ch<sub>2)<sub>4</sub>C(OH)(C<sub>6</sub>H<sub>11</sub>-n),</ch<sub>	9.1	20.7	2.3
		5.3	7.2	1.4
		6.7	13.6	2.0
		6.4	12.8	2.0
266	-Cyclohexyl	7.6	4.04	0.53
2328	-cis-β-Decalyl		2.3	.3*
1916	-( <ch<sub>2)<sub>4</sub>-Cyclohexyl (standardization)</ch<sub>	8.2, 7.3		1.0
Average.....		7.4		

\* Calculated with use of the average value of A.

V summarizes the results. The ratios reported in the last column give a measure of the susceptibility, relative to M-1916, to antagonism in a medium containing 16 per cent human serum as compared to that in pure duck serum. The thirteen separate determinations of the ratio of  $IC_{50}$  values for M-1916 show that the

results are fairly reproducible. The data for the seven compounds studied illustrate a point that has been demonstrated more fully by Wendel, namely that the ratio of susceptibility to human and duck plasma protein is by no means constant for the naphthoquinone series. Thus the extreme variation in the ratio B/A is from 0.2 to 4.5, or 1:22.5. In order to produce 50 per cent inhibition in the mixed serum, M-1916 must be taken in 7.4 times the concentration required in pure duck serum, and Wendel finds that a 25-fold increase in concentration is required in a cell suspension in pure human serum. Thus the standard drug is subject to considerable human-protein antagonism, but some of the compounds listed in table V are still more susceptible. M-2293, with a ratio of 0.2,

TABLE VI

*Effect of human serum on the antirespiratory power of M-2336*

(1.5 cc. cell suspension in pure duck serum, 0.2 cc. drug solution, 0.3 cc. adjusted duck serum or adjusted human serum).

SERUM	CONCENTRATION		INHIBITION per cent	$IC_{50} \times 10^{-1}$ moles per liter
	M-1916	M-2336		
Duck	0.8	mgm. per liter 11.7 2.9	62.1	21.7 225
	.4		23.2	
	.2		10.6	
			62.3	
			28.5	
Human-Duck		187	71.4	1080
		93.5	71.6	
		46.7	69.6	
		23.4	1.8	
		11.7	0	
		5.8	0	

is less subject to antagonism by human plasma proteins than any other compound studied; it shows about the same activity in the presence of human and duck sera.

Table VI reports a study of the human-protein susceptibility of M-2336, the 4'-hydroxy derivative of M-1916 identical with one of the two isolated products of metabolism. The value found for  $IC_{50}^{human}/IC_{50}^{duck}$  is 4.8, and the ratio of this to the average value for M-1916 is B/A = 0.65. Thus this metabolite has rather favorable characteristics with respect to human-protein susceptibility, and this may have contributed to the mild therapeutic action of M-1916. A peculiarity is discernible in the dosage-response relationship for M-2335 given in the last section of table VI. Whereas ordinarily a doubling of the dose causes a two- or three-fold increase in inhibition, an increase from 23.4 to 46.7 mgm./l. here caused a forty-fold increase.

*Nature of Drug Action*—The antagonistic power of human serum was utilized in an experiment designed to determine whether the inhibition of respiration is

reversible (table VII). Graded concentrations of M-1916 were added to three series (A, B, C) of Warburg vessels containing a parasitized cell suspension in duck serum. To Series A human serum was added and mixed with the suspension. Series B and C contained an equal amount of human serum in the side vessel of the Warburg flasks and the flasks were inverted after 60 and 120 minutes, respectively. The concentrations were so chosen that all the suspensions in Series B and C were completely inhibited before inversion. Thus the cells in Series B were exposed for one hour to high concentrations of M-1916, after which the inhibition was weakened by addition of human serum, and a dose-response curve was obtained that did not differ markedly from the curve pertaining to Series A during the second hour. However, when the human serum was mixed with the cell suspension in Series C, which had been exposed for two hours to high

TABLE VII

*Effect of the addition of human serum on the respiration of cell suspensions in duck serum maximally inhibited by M-1916*

SERIES	HUMAN SERUM ADDED	1ST HOUR		2ND HOUR		3RD HOUR	
		IC <sub>50</sub> (10 <sup>-7</sup> M)	Rel. potency	IC <sub>50</sub> (10 <sup>-7</sup> M)	Rel. potency	IC <sub>50</sub> (10 <sup>-7</sup> M)	Rel. potency
A	None	11.9	1.0	9.7	1.0	7.7	1.0
	At start	119	0.1	78	0.12	62.0	0.12
B	In 60 min.			61	.16	42.5	.18
C	In 120 min.					32.5	.24

Final pH in all drug flasks: 7.31-7.40

		1ST 45 MINS.		2ND 45 MINS.		
		IC <sub>50</sub> (10 <sup>-7</sup> M)	Rel. potency	IC <sub>50</sub> (10 <sup>-7</sup> M)	Rel. potency	
A'	None	11.8	1.0	9.0	1.0	
B'	At start	101	0.12	52	0.17	
	In 45 min.			47	.19	

Final pH in all drug flasks: 7.21-7.32

concentrations of M-1916, respiration remained low compared with the respiration during the third hour of the cells in Series A and B. The parasites recover fairly well after exposure to drug for one hour but seem to be more seriously affected on exposure for two hours. The observation provides some indication that the lethal action of naphthoquinone drugs on trophozoites may, indeed, be associated with the impairment of parasite respiration.

*Effect of Various Additives*—Preliminary experiments seemed to indicate a synergistic or antagonistic relation between naphthoquinone drugs and compounds of the nature of the degradation products isolated from biological fluids. To confirm or disprove these experiments, we measured the antirespiratory power of a number of equimolar mixtures prepared from an active naphthoquinone drug and a compound of the type of the metabolites isolated; i.e. having the side chain  $-(CH_2)_8C(OH)(CH_2)_2$  (M-2231) or  $-CH_2CH_2CH_2CH(CH_3)COOH$  ("Quinone Acid"). In one case a solution of M-2279 was mixed with an equimolar solution

of quinone pigments extracted from the plasma of a patient receiving M-2279. The results are given in table VIII. The  $IC_{50}$  value A refers to the antirespiratory power of the pure drug; B is the concentration at which the same drug produced 50 per cent inhibition in the presence of an equimolar amount of a

TABLE VIII  
*Effect of added quinones on the  $IC_{50}$  of M-1916 or M-2279*

DRUG	SUBSTANCE ADDED	$IC_{50}$ ( $10^{-7}$ M)		RATIO A/B	
		A Drug	B Mixture	Calcd.	Found
M-1916	M-2231	19.8	10.0	1.2	2.0
		21.6	7.9	1.2	2.7
		7.1	5.1	1.2	1.4
		11.5	10.6	1.2	1.1
		15.0	11.0	1.2	1.4
		12.2	9.8	1.2	1.3
		19.3	16.7	1.2	1.2
		24.0	16.2	1.2	1.5
		3.7	9.3	1.05	0.4
		3.3	8.3	1.05	.4
		5.1	5.9	1.05	.9
		2.6	3.4	1.05	.8
M-1916	Quinone acid	19.3	23.0	1.0	.8
M-2279		24.0	19.8	1.0	1.2
		8.3	5.5	1.0	1.5
M-2279	Naphthoquinone plasma pigments after drug administration	10.0	5.3	1.4	1.9

TABLE IX

*Failure of vitamin K<sub>1</sub>, carotene, or quinine to influence respiration or inhibition by M-1916 of duck erythrocytes infected with P. lophuriae*

ADDED SUBSTANCE CONCENTRATION	NONE	CAROTENE, $9$ MG.M./L.	VITAMIN K <sub>1</sub>			QUININE HYDROCHLORIDE			
			None	17.9 mgm./L.	35.7 mgm./L.	None	$10^{-6}$	$10^{-5}$	$10^{-4}$
Respiration, cmm./hr.....	270.5	268	305	296	294	232	227	209	213
$IC_{50}$ , $10^{-7}$ M.....	19.7	18.6	7.5	6.0		11.1	11.4		

given added substance. The relative antirespiratory power of each of the three added substances is known, and thus calculation can be made of the activity the mixture should have if there were no synergism or antagonism. As the table shows, the ratio A/B does not deviate from the calculated value in a systematic or reproducible way.

We also studied carotene, vitamin K<sub>1</sub> (as colloidal solutions in saline), and

quinine for possible synergistic or antagonistic effect on quinones. The results appear in table IX; no effect was observed.

Another experiment (table X) was made to see if a bile acid might tend to break up the drug-protein complex and release more substance for drug action. At a concentration as high as 780 mgm./l. sodium glycocholate has little effect on cell respiration or on the antirespiratory power of M-1916 (a), and has only a slight effect in relieving antagonism by human plasma proteins (b).

TABLE X  
*Effect of sodium glycocholate*  
(a) On the respiration in pure duck serum

M-1916	RESPIRATION IN CCM./HR. AT THE FOLLOWING CONCENTRATIONS OF ADDITIVE (MG.M./L.):			
	0	7800	780	78
mgm./l.				
0	201	40	172	198
0.8		29	73	76
.4		31	126	129
.2		30	146	143

(b) On the human-protein antagonism to M-1916 (1.5 cc. of cell suspension in duck serum + 0.3 cc. of human serum)

Per cent inhibition

M-1916	DUCK SERUM	HUMAN-DUCK SERUM	HUMAN-DUCK SERUM + 780 MG.M./L. ADDITIVE
mgm./l.			
6.4		68	
3.2		53	
1.6		34	
0.8	65.6		76
.4	54.2		67
.2	31.3		53
IC <sub>50</sub> (10 <sup>-7</sup> M)	11.7	95.0	46.0

#### SUMMARY

1. Satisfactory methods have been found for the accurate assay of naphthoquinone pigment in plasma samples and for the preparation of plasma extracts (alcohol) suitable for respiratory study.

2. Difficulties encountered in ether extraction are attributable to the binding of naphthoquinone to protein. Exploratory dialysis and solubility experiments show that the compounds are bound particularly to albumin; a typical naphthoquinone-albumin complex has an apparent acidity about 1.6 pK units lower than that of the free naphthoquinone. The drug-binding power of three plasmas, as judged by dialysis experiments, is in the order: human > monkey > duck.

3. Little if any naphthoquinone diffuses from the plasma into the red blood cells.
4. Protein antagonism to drug action as measured by the depression of the inhibitory action of naphthoquinones on the respiration of parasitized erythrocytes, decreases in the order: human serum > chicken serum > duck serum; the difference in protein antagonism between chicken and duck plasma may account for marked differences noted in the antimalarial potency of naphthoquinones in these species.

5. Susceptibility to human-protein antagonism varies markedly from compound to compound, and the characterization of naphthoquinones with respect to this factor affords a further criterion for the laboratory evaluation of their promise in therapy.

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# NAPHTHOQUINONE ANTIMALARIALS

## XX. METABOLIC DEGRADATION<sup>1</sup>

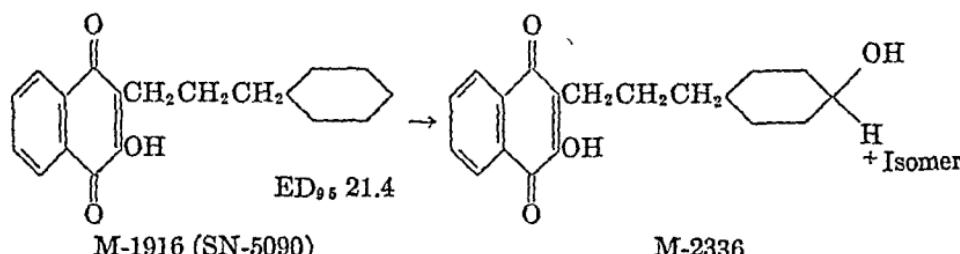
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The isolation and chemical characterization of degradation products of naphthoquinone antimalarials administered to human subjects (Paper XVIII) has established the nature of the metabolic processes resulting in drug deactivation and has provided a clue to the development of improved drugs that is the chief subject of the present investigation of the rate and extent of metabolic deactivation by determination of the antirespiratory activity of plasma pigment. The present method is applicable also to interesting members of the series not easily studied by the previous method because of limited urinary excretion of metabolites.

The key to the advancement of the problem is that M-1916, which is only one of the more moderately potent members of the series against avian malarial infections,<sup>3</sup> exhibited a positive though temporary therapeutic effect in man (1)



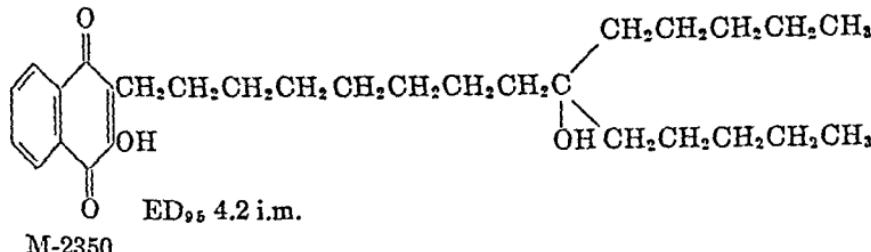
that seems attributable to the action of two alcoholic metabolites that retain 8-10 per cent of the antirespiratory activity of M-1916 and that persist in the blood for long periods. The observation suggested that a synthetic naphthoquinone with an already hydroxylated side chain might be more resistant to metabolic alteration than the compounds with hydrocarbon side chains. The introduction of a hydroxyl group into the parent compounds with C<sub>9</sub>-C<sub>13</sub> side chains is attended with a marked drop in activity. A study of the relative distribution characteristics (Paper XV) showed that for optimum antimarial activity a certain balance between lipophilic and hydrophilic character is re-

<sup>1</sup> See Paper XVIII (This Journal) for acknowledgements to the Committee on Medical Research and the Rockefeller Foundation.

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<sup>3</sup> The results of assays against *P. lophurae* in ducks (A. P. Richardson, ref. 1) are expressed in terms of the Effective Dose in milligrams per kilogram (three doses per day) required to produce a 95% reduction in parasitemia; administration was oral except as noted.

quired and that hydroxylation of the side chain is attended with a very marked hydrophilic displacement. A compensating shift in the opposite direction can be achieved by an increase in the size of the hydrocarbon side chain. These considerations prompted the synthesis of compounds of a new type, of which M-2350 is the most promising present example. This substance, with a hydroxylated



$\text{C}_{19}$ -side chain, has the proper distribution characteristics and it shows high potency in duck assays on intramuscular administration, though not when given orally. The chief purpose of the present investigation was to explore the rate and extent of drug degradation of already documented compounds and to evaluate M-2350 by comparison of its performance. The results have been sufficiently encouraging to warrant initiation of a clinical trial for curative action at the Medical School of the American University in Beirut.

**METHOD.** Observations by Wendel (2) and by us (Paper XXII) have established the validity of accepting activity in the inhibition of respiration of parasitized erythrocytes of ducks (*P. lophurae*) as a reliable measure of antimalarial activity. Hence the course of drug degradation in human subjects was followed by determination of the anti-respiratory activity per colorimetric equivalent of naphthoquinone pigment extracted from plasma samples by alcohol precipitation of proteins. The activity is expressed as the percentage of the activity of the compound administered, redetermined each time in a parallel run. The colorimetric assays are all corrected for native plasma pigment.

In most of the experiments a solution for intravenous administration was prepared by dissolving 0.6-1 gram of naphthoquinone and 200 mgm. of sodium hydroxide in 20-50 cc. of alcohol and 50-100 cc. of saline and diluting the red solution with 1400 cc. of saline and 100 cc. of 25 per cent salt-free human albumin solution. An improved procedure later developed for M-2350 is as follows: 2 grams of M-2350 is dissolved in 50 cc. of alcohol and the solution is added all at once with agitation to a solution of 1.2 grams of sodium carbonate ( $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ ) and 200 cc. of 6 per cent Knox pyrogen-free gelatin solution (pH 7.4) in 1500 cc. of physiological saline; the red colloidal solution is at once autoclaved for 20 minutes. No toxic reactions or changes in blood NPN or blood counts were seen in patients receiving this solution intravenously. The injection usually was accomplished in periods of from one and one-half to two and one-half hours; blood samples were drawn for analysis at the indicated times subsequent to the completion of the injection.

**RESULTS.** The results of the study of the rate and extent of metabolic deactivation of seven naphthoquinones are recorded in table I. The results for M-1916 confirm and supplement the findings cited above. The substance is completely degraded within about four hours to material possessing one-tenth the original activity and this material is retained in the blood at a substantial level for a remarkably long time. The mild therapeutic effect noted thus is

undoubtedly due to the hydroxylated metabolites. The highly potent M-2293 is degraded about as rapidly and extensively; the degraded pigment is much more

TABLE I  
*Metabolism in man*

M-	NAPHTHOQUINONE		DOSE	PLASMA PIGMENT			
	Side chain	ED <sub>50</sub>		Time	Level	Activity	
				hours	mgm. %	per cent	
1916	-(CH <sub>2</sub> ) <sub>3</sub> -Cyclohexyl SN-5949	21.4	0.6 i.v.	0.5	7.4	33	
				1.5	6.5	21	
				4	5.4	13	
				20	4.2	10	
2293	4-Cyclohexylcyclohexyl SN-13336	0.7	0.6 i.v.	0.5	3.6	27	
				1.5	3.3	10-15	
				5	2.4	8	
				16	0.9		
			3.0/day p.o.	15	0.4	feeble	
				24	0.4	0	
266	Cyclohexyl SN-5094	8.0	1.0 i.v.	0.5	6.0	84	
				1.5	4.5	77	
				3	3.4	49	
				6	2.9	42	
			0.6 i.v.	0.5	4.0	48	
				3	3.0	23	
				5	2.0	16	
			2.0/day for 3 days, p.o.	8	2.0	25	
				24	5.5	30	
				48	6.0	22	
				72	4.9	27	
2301	-(CH <sub>2</sub> ) <sub>6</sub> -C <sub>6</sub> H <sub>5</sub> SN-13451	13.9	1.0 i.v.	0.5	8.4	62	
				1.5	6.5	63	
				3	4.8	42	
				6	2.3	40	
				16.2	2.2	13	
2289	-(CH <sub>2</sub> ) <sub>3</sub> C <sub>6</sub> H <sub>4</sub> Cl-p SN-12855	18.1	0.6 i.v.	0.2	2.4	92	
				0.5	1.9	77	
				1	1.5	10	
			1.2 i.v.	0.5	6.8	95	
				1.5	5.8	98	
				4	2.4	59	
			3.0/first day, 1.0/day next 2 days, p.o.	24	0.6	24-65	
				48	0.3		
				72	0.5	55	

TABLE I—Continued

M-	NAPHTHOQUINONE	ED <sub>50</sub>	DOSE	PLASMA PIGMENT		
				Time	Level mgm. %	Activity per cent
2309	$-(CH_2)_3C_6H_4OC_6H_5-p$ SN-13936	7.2 IM	0.6 i.v.     	0.5	10.2	100
				1.5	8.7	79
				3	6.9	66
				5	6.3	55
				15	4.1	28
			1.0 i.v.	0.5	6.0	84
				1.5	4.5	77
				3	3.4	49
				6	2.9	42
			0.6 i.v.	0.5	3.6	97
				1.5	2.9	81
				5	1.8	60
				20	0.9	24
			2.0/day for 3 days, p.o.	12	2.2	96
				24	3.8	77
				36	4.0	63
				60	3.1	56
2350	$-(CH_2)_3C(OH)(C_6H_{11}-n)_2$	4.2 IM	0.6 i.v.     	0.5	6.8	104
				1.5	5.1	96
				3	4.1	67
				5	3.3	48
				20	1.5	26
			0.6 i.v.	0.5	7.5	>90
				1.5	5.4	99
				3	3.9	104
				5	2.8	88
				20	1.1	81
			2/4 hrs., i.v.	6	4.0	130
				24	1.3	17
			2/4 hrs., i.v.	5	10.0	90
				9	4.3	100
				42	2.7	55
			0.6/8 hrs., p.o.	13	2.6	40
				26	2.1	18
				49	2.2	22

active than that from M-1916, but it is eliminated much more rapidly. This consideration, coupled with the poor performance on oral administration, offers little promise in therapy in spite of the superior

potency in bird assays. The cyclohexyl derivative, M-266, when administered intravenously is degraded about as rapidly and extensively as M-1916; deactivation was also extensive when the compound was given by mouth. The residual activity here also can be attributed to a hydroxylated metabolite. The  $\omega$ -phenylnonylderivative, M-2301, and the *p*-chlorophenylethyl compound, M-2289, seem to retain activity for slightly longer periods when given intravenously, but extensive degradation eventually occurs in both instances, and the second substance performed very poorly when given by mouth.

The phenoxyphenylalkyl side chain of M-2309 seems more resistant to degradation than purely hydrocarbon side chains, possibly because of the presence of oxygen in ether linkage. Compound M-2350, with a hydroxylated side chain, performed even better. The results of the four intravenous experiments are variable but indicate a definite trend to better retention of activity (see also below). The one oral experiment reported is not encouraging, and intramuscular injection (2 grams of M-2350 plus 6 cc. of nut oil) was tried without promise (brief local pain, plasma levels in three instances in the range of only 1-2 mgm. per cent). Both these experiences and the results of assays in ducks (low activity on oral administration) indicate that this substance of high molecular weight is not absorbed efficiently from the gut and has potentialities as a therapeutic agent only if administered parenterally.

The *cis*- $\beta$ -decalylpropyl derivative, M-2279 (SN-12320), is of interest because of the finding of Walker and Richardson (3, 4) that this naphthoquinone potentiates Pamaquine in both the suppression and cure of *P. cathemerium* in ducks. Table II gives the results obtained on oral administration of the compound to one subject in daily doses and to another in a discontinuous regime. No consistent differences are discernible, and the average plasma level of effective drug (last column) is much the same: 3.4 and 3.0 mgm. per cent. The overall average activities are 37 and 47 per cent that of the administered material; the average of thirteen determinations in four cases not reported was 40 per cent. The urinary excretion of pigment derived from M-2279 is too low to permit chemical characterization, but the pigment derived from a large plasma specimen from a polycythemic subject was investigated and found to be extractable from ether with sodium carbonate solution whereas M-2279 is not. The substance very probably is metabolized to an alcoholic derivative. If, as the results suggest, this possesses 40 per cent of the activity of the highly potent M-2279, it should offer promise as a drug.

*Parenteral Administration to Various Test Animals.* The first objective of the exploratory experiments of table III was to find a test animal showing a response to naphthoquinone administration comparable to that of man and therefore suitable for the preliminary evaluation of new members of the series with respect to persistence and resistance to deactivation. Comparisons were made of the fate of M-1916 and M-2350, compounds that are susceptible, and relatively resistant, respectively, to deactivation in the human body. The parenteral route was selected in order to eliminate the variable factor of absorption, and a convenient medium for injection was found in a heat-stable solution of naphtho-

quinone sodium salt in 5 per cent gelatin. The dose was the same as in the trials in man (table I), namely 10 mgm./kgm.

Intravenously administered M-1916 disappears from the blood of dogs, cats, and rabbits in a matter of a few minutes after the injection. M-2350 is eliminated from the blood of the dog and the rabbit at a significantly slower rate. M-1916 persisted for a somewhat longer period in the guinea pig than in the above animals, but no side-chain degradation was noted in the rather brief period

TABLE II  
*Metabolism in man*  
M-2279 (SN-12320): -(CH<sub>2</sub>)<sub>4</sub>-β-Decalyl-cis, ED<sub>50</sub> 3.5

DAY	ORAL DOSE	PLASMA PIGMENT		EFFECTIVE DRUG LEVEL mgm. %
		Mgm. %	Activity %	
	gm.			
1	3	5.1	64	3.3
2	2	4.9	30	1.5
3	2	4.9	24	1.2
4	1	8.7	44	3.8
5		10.3	58	5.9
6	2	10.1	33	3.3
7	1	12.3	47	5.8
8	1	11.4	37	4.2
9	1			
10	1	11.8	27	3.2
11	1			
12	1			
13		10.4	7.5	0.8
1	3	6.1	49	3.3
2		4.3	81	3.5
3		3.7	45	1.7
4	3	3.5	36	1.3
5		9.2	73	6.7
6		7.6	41.5	3.2
8	3	5.9	24	1.4
9		9.2	30	2.8
10		8.1	38	3.1
11		6.9	40	2.8

during which an effective concentration of pigment was found in the plasma. An anesthetized monkey gave a much better response with respect to persistence of drug, but again very little drug degradation occurred. Intravenously injected M-1916 persisted in the blood of a duck for more than one hour and suffered no degradation.

The mouse proved to be a particularly satisfactory test animal. In order to obtain sufficient plasma for the determination of plasma concentration and anti-respiratory activity it is necessary to use pooled blood (by decapitation) of several

TABLE III

*Parenteral administration of naphthoquinones*

(Standard dose = 10 mgm./kgm. as sodium salt in 5% gelatin solution\*)

QUINONE	ANIMAL	INJECTION		PLASMA SAMPLE		
		ROUTE	MIN.	TIME	MGM. %	ACTIVITY
M-1916	Dog	i.v.	0.5	min.		
				1	3.55	
				3	2.03	
				6	1.21	
				12	0.50	
M-2350	Dog	i.v.	2	2	4.01	
				5	2.65	
				20	1.57	
				46	0.14	
M-1916	Cat	i.v.	0.5	2.5	2.07	
				4.5	1.72	
				9.5	0.94	
				20	0.86	
M-1916	Rabbit	i.v.	1	3	3.59	
				6	2.81	
				12	1.91	
				24	0.11	
M-2350	Rabbit	i.v.	1	4	2.53	
				15	1.18	
				45	1.02	
				120	0.65	
M-1916	Guinea pig	i.v.	0.5	4	4.38	
				35	1.49	
				55	0.30	
				85	0.13	100
M-1916	Rhesus monkey (under nembutal anesthesia)	i.v.	7	15	4.34	
				70	2.38	
				200	1.07	94
				300	0.90	
M-1916	Duck†	i.v.	12	20	1.70	90
				80	0.55	120
M-1916	White mice: 10 5 5 5	i.v. i.p. in nut oil‡		60	0.83	37
				120	0.28	
				3 hrs.	3.93	
				6 hrs.	2.58	
				24 hrs.	0.18	

TABLE III—Continued

QUINONE	ANIMAL	INJECTION		PLASMA SAMPLE		
		Route	Min.	Time min.	Mgm. %	Activity
					%	
M-2350	White mice: 5 5 10 5  5 10 10  10 5 10 5	i.v.		15	1.17	
				90	0.64	
				195	.47	54
				300	.07	
		i.p.		60	0.85	
				195	.47	89.5
				420	.23	
		i.p. in nut oil		3 hrs.	2.14	
				7 hrs.	1.79	
				15.5 hrs.	1.82	40
				24 hrs.	0.29	

\* The solution was prepared by dissolving 20 mgm. of the quinone and about 70 mgm. (one-half pellet) of sodium hydroxide in 25 cc. of 5% gelatin solution; the mixture can be warmed without damage to the protein and a clear red solution results.

† The solution injected contained 23.5 mgm. of M-1916 dissolved in 50 cc. of a solvent consisting of 80% duck serum, 18% saline, and 2% ethanol (experiment by H. Heymann).

‡ A solution of 50 mgm./cc. of naphthoquinone in nut oil (Inventol); the dose injected was 0.1 cc. per mouse.

animals in each experiment, but this has the advantage of providing data based upon averages. The results recorded in table III show that intravenously injected (tail vein) M-1916 and M-2350 both persist in the blood for considerable periods and suffer metabolic deactivation comparable to that observed in man; M-2350 persists better and is degraded less extensively than M-1916.\*

A comparison was then made of different methods of administering naphthoquinones to mice. The intraperitoneal injection of M-2350 in gelatin solution in the standard dose of 10 mgm./kgm. resulted in substantial plasma levels persisting for several hours and with high retention of the original activity. The injection into the peritoneum of a very large dose (150 mgm./kgm.) of M-1916 or M-2350 in nut oil was then tried and found to constitute an efficient method of maintaining a high concentration of naphthoquinone pigment in the blood over a considerable period of time. The pigment present in mouse plasma at a level of 1.82 mgm. per cent fifteen hours after administration of M-2350 by this technique still retained 40 per cent of the original activity.

*Drug Distribution.* In one of the cases studied a man with a total biliary fistula was given 1.1 grams of M-1971 by intravenous injection and the bile was collected during the subsequent twenty hours. No trace of naphthoquinone could be detected in the bile sample.

\* Plasma samples from rats given M-1916 orally were kindly sent to us by Dr. L. H. Schmidt; our analyses indicate that the rat likewise effects metabolic oxidation of the naphthoquinone side chain.

*Site of Drug Metabolism.* Two independent observations furnish evidence that the metabolic oxidation of the side chain occurs elsewhere than in the intestinal tract. Incubations of M-1523, M-1916 and M-285 with *Alcaligenes faecalis*, *E. coli*, and mixed human intestinal flora were kindly prepared by Dr. L. H. Schmidt and brought to Cambridge for examination. Fractional extraction with buffers afforded no indication of any degradation of the type observed on administration of the compounds to human subjects. The second piece of evidence is the already recorded fact that intravenously administered M-1916 suffers the same oxidative degradation as orally administered material.

A first attempt to effect drug metabolism *in vitro* was conducted as follows. A mixture of 250 mgm. of finely ground M-285, 100 cc. of a 10 per cent solution of crystalline bovine albumin in physiological salt solution, and 300 mgm. of sodium carbonate was shaken for several hours and the red solution filtered from 20–25 mgm. of undissolved quinone and homogenized in a Waring blender with 286 grams of liver freshly removed from a dog and 25,000 units of penicillin. The resulting pasty mass was then incubated at 38° for forty-five hours in an

TABLE IV  
*In vitro degradation of M-1916 by mouse liver*

TIME OF INCUBATION	NAPHTHOQUINONE RECOVERED	
	Per cent	Per cent Activity
hr.		
1	80	100
2	78	84
3	66	69
6	50	58

atmosphere of 90 per cent oxygen and 10 per cent carbon dioxide, with frequent mechanical agitation. The greenish brown semisolid mass was shaken with successive portions of ether and water and the pigment was extracted from the combined ether extracts and determined colorimetrically. The total recovery was 62 mgm. (28 per cent), and buffer fractionation showed that the naphthoquinone remaining was all completely undegraded. Thus the enzymatic oxidation in the presence of dog liver resulted in the conversion of three-quarters of the starting material to non-quinonoid products and effected no attack of the side chain. The observation is in harmony with the very rapid destruction of naphthoquinone administered to dogs by intravenous injection.

The findings recorded in table III suggested a repetition of the experiment utilizing mouse liver, since the intact mouse is capable of degrading the naphthoquinones. In each experiment recorded in table IV the livers from five white mice were homogenized with a small volume of saline and mixed with a solution of 1.5 mgm. of M-1916 in 2.5 cc. of a 20 per cent gelatin solution containing 10 mgm. each of sodium carbonate and sodium bicarbonate. The mixture was then incubated at 38° in a stream of oxygen containing 5–10 per cent carbon dioxide

for varying periods of time, after which alcohol was added. The mixture was centrifuged, and the naphthoquinone pigment left on evaporation of the alcoholic extract was assayed and its antirespiratory activity determined. The results demonstrate a progressive loss in activity of the naphthoquinone pigment comparable to that observed in intact mice and in man, and the conclusion may be drawn that in this series of compounds metabolic drug degradation occurs in the liver.

*Toxicity for Mice.* Those naphthoquinones that appeared of interest for inclusion in the metabolism studies but that had not been examined by pharmacologists in the CMR organization were probed by oral and intravenous tests. The second test revealed marked toxicity in some compounds that probably appeared innocuous in the oral test merely because they are too sparingly soluble to be effectively absorbed. We are greatly indebted to Dr. Otto Krayer and to Dr. E. B. Astwood for short-term chronic toxicity tests in mice conducted in the Department of Pharmacology of the Harvard Medical School. The overall summary recorded in table V reveals striking differences among structurally related compounds and gives some index of the limits of safety in the experimentation with these substances. The compounds are listed in the order of increasing mortality noted, although the gain or loss in weight and the amount of food consumed also constitute useful criteria. Thus M-298 and M-333 were excluded from metabolism studies because of the adverse showing on all these counts. In most of the experiments a parallel test was made of M-1916 as standard.

*Intravenous Toxicity for Dogs.* Richardson (1, 5) observed that M-1916, M-297, and M-1971 all produced shock, and sometimes death, when a dose of 25 mgm./kgm. was given intravenously to a dog in seven minutes in a 1 per cent solution prepared by heating 500 mgm. of quinone and 100 mgm. of sodium hydroxide with 40 cc. of water and adding 10 cc. of alcohol. In a first trial with M-1916 the dose, time of injection, and amount of alkali were the same as in Richardson's procedure but the drug was brought into solution not by adding alcohol but by using a suitably large volume of hot saline such that the concentration of drug was one-tenth that in Richardson's experiment (experiment 1, table VI); no abnormal symptoms were observed (blood pressure 115 mm. Hg). When Richardson's procedure was followed exactly his results were duplicated (experiment 2), for the dog went into shock (blood pressure 60 mm. Hg). The standard dose was again given in a dilute solution (0.2 per cent) and followed by the injection of 30 cc. of 20 per cent alcohol, and again symptoms (other than incoordination) were absent. The injection of 10 per cent alcohol containing alkali but no drug likewise failed to produce shock. The contrast between the innocuous character of a large volume of a 0.1-0.2 per cent solution of drug and the toxic effect of a small volume of a 10 per cent solution was then confirmed by consecutive injections in the same dog (experiment 5). It seems possible that drug entering the blood stream in a small volume of a relatively concentrated solution may not be taken up by protein at the rate at which it is introduced and is hence present in part in the free condition rather than in the form of a presumably less toxic protein complex. This hypothesis is supported by the observation that shock

did not result after the injection of more than a full dose of 1 per cent solution of M-1916 prepared by dissolving the drug with either sodium carbonate or sodium hydroxide in 50 cc. of a 10 per cent solution of crystalline bovine albumin containing 10-20 per cent of alcohol (experiments 7 and 8). The shock-producing action of a concentrated solution is thus counteracted by providing protein with which the drug can combine prior to injection. A protein is an advantageous

TABLE V  
Toxicity for mice

(Averages for 10 mice at each of the following dosages for 7 days: 1, 0.5 and 0.25% drug in diet)

M-	SIDE CHAIN	CHANGE IN WT.	FOOD	DEATHS
			gm./kgm./ day	
	Controls (150 mice)	+3.3	223	0
2256	-C <sub>17</sub> H <sub>35</sub> -n	+4.6	276	0
1714	-C <sub>15</sub> H <sub>31</sub> -n	+4.0	254	0
2293	-4'-Cyclohexylcyclohexyl	+2.0	180	0
344	-CH <sub>2</sub> -Piperidyl	+1.4	285	0
2350	-(CH <sub>2</sub> ) <sub>8</sub> C(OH)(C <sub>6</sub> H <sub>11</sub> -n) <sub>2</sub>	+0.1	126	0
2289	-CH <sub>2</sub> CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> Cl-p	-2.0	118	0
1960	-β-Naphthyl	-0.1	156	3
1929	-(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	+1.0	175	7
295	-(CH <sub>2</sub> ) <sub>2</sub> -β-Tetralyl	+0.8	188	13
1916	-(CH <sub>2</sub> ) <sub>2</sub> -Cyclohexyl (180 mice)	+0.4	168	15
2333	-(CH <sub>2</sub> ) <sub>2</sub> -Δ <sup>2</sup> -Cyclohexenyl	-1.3	153	20
266	-Cyclohexyl	+2.1	312	23
380	-(CH <sub>2</sub> ) <sub>2</sub> -4'-Cyclohexylcyclohexyl-trans	-1.4	116	23
2284	-(CH <sub>2</sub> ) <sub>6</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	-2.7	99	23
297	-(CH <sub>2</sub> ) <sub>2</sub> -β-Decalyl-trans	-2.1	77	25
2279	-(CH <sub>2</sub> ) <sub>2</sub> -β-Decalyl-cis	-2.0	84	27
2301	-(CH <sub>2</sub> ) <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>	-1.3	101	30
1523	-(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	-1.6	94	30
2309	-(CH <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>4</sub> OC <sub>6</sub> H <sub>5</sub> -p	-2.4	160	50
2287	-(CH <sub>2</sub> ) <sub>8</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	-3.0	75	57
2260	-(CH <sub>2</sub> ) <sub>4</sub> C <sub>6</sub> H <sub>4</sub> Cl-p	+1.7	240	60
2244	-(CH <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>4</sub> F-p	-1.6	113	60
374	-(CH <sub>2</sub> ) <sub>2</sub> -Δ <sup>2</sup> -Cyclohexenyl	+1.3	173	80
298	-CH <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>2</sub> CH(C <sub>2</sub> H <sub>5</sub> )C <sub>6</sub> H <sub>4</sub> -n	-1.6	72	83
333	-CH <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	-1.8	115	90

component of a solution for intravenous injection both because of its detoxifying action and because of its solvent power for the naphthoquinones.

The following procedure was adopted for the study of the intravenous toxicity of other members of the series. A 0.1 per cent solution of drug (500 mgm.) and sodium hydroxide (100 mgm.) was prepared in saline, or in saline containing alcohol if required for solution, and a portion of the solution corresponding to a dose of 25 mgm./kgm. was injected in a period as close as possible to seven minutes.

The following compounds produced no abnormal symptoms and the dogs survived: M-1929, M-295, M-374, M-344, M-266, M-2260, M-1523, M-297, M-380, M-2293, M-2289, M-2309, M-2301, M-2350. When given in the same way M-333 killed each of two dogs; since a dangerous degree of toxicity was observed both in this test and in the test in mice the compound was excluded from metabolism study.

In the case of four compounds of high molecular weight and low solubility, rather high concentrations of alcohol were required for solution and this may have potentiated the toxicity. The test solutions of M-1714 and M-2256 (-C<sub>16</sub>H<sub>31</sub>-n and -C<sub>17</sub>H<sub>35</sub>-n) contained 28 per cent of alcohol and produced shock and then death. In a second experiment with M-1714 a 500-mgm. sample was dissolved with 100 mgm. of sodium hydroxide in 230 cc. of a 5 per cent solution of crystalline bovine albumin and 20 cc. of alcohol; a full dose (160 cc.) injected into a dog produced no symptoms. M-2293 (4'-cyclohexylcyclohexyl) was likewise in-

TABLE VI  
*Intravenous (7 min.) administration of M-1916 to dogs*

EXPT.	SOLUTION INJECTED				SYMPTOMS
	M-1916	Alcohol	Base	Albumin	
	%	%		%	
1	0.1	0	NaOH	0	None
2	1.0	19	NaOH	0	Shock
3	0.2	4	NaOH	0	None
4	0	10	NaOH	0	None
5a	0.2	4	NaOH	0	None
5b	1.0	20	NaOH	0	Death
6	0.2	20	NaOH	0	None
7	1.0	10	Na <sub>2</sub> CO <sub>3</sub>	10	None
8	1.0	20	NaOH	10	None

corporated into a test solution containing 28 per cent of alcohol, and this caused the death, by peripheral circulatory collapse, of each of three dogs even though in two instances 5-10 per cent of bovine albumin was added to the test solution. An alcohol-free solution was then prepared by dissolving a full dose of drug plus 200 mgm. of sodium hydroxide in boiling water at a concentration of 0.09 per cent, cooling the solution to 40°, and adding 10 per cent of bovine albumin. The injection of this solution produced some dyspnea and retching but the dog remained conscious and soon recovered. Exactly comparable results were obtained with the higher homolog M-380; a solution containing 19 per cent alcohol proved lethal, while an alcohol-free solution containing albumin produced only temporary symptoms.

#### SUMMARY

1. A method of evaluating naphthoquinones as possible antimalarial drugs with respect to plasma levels, extent of metabolic deactivation, and persistence of

active material following oral or intravenous administration to nonmalarial individuals consists in determination of the antirespiratory activity per colorimetric unit of total naphthoquinone pigment extracted from plasma samples drawn at varying periods of time.

2. Compounds with hydrocarbon side chains terminating in a ring are rapidly metabolized to products hydroxylated in this ring. The metabolites retain 10-60 per cent of the original activity, often persist in the blood for long periods, and in some instances may exert a therapeutic effect.

3. A synthetic compound having a hydroxyl group in a large hydrocarbon side chain shows improved resistance to metabolic deactivation, possesses adequate activity (i.v.), and appears to offer promise as a drug.

4. Of various animals investigated for the laboratory evaluation of new compounds, the mouse shows the closest parallelism to man in the retention and degradation of the naphthoquinones. Drug degradation *in vitro* was accomplished in a mouse liver homogenate.

5. Intravenous toxicity observed in dogs under certain conditions can be counteracted by adding albumin to the solution injected.

We wish to thank Sally S. Shy, Shirley R. Katz, and Grace Nahm for technical assistance in the respiratory studies.

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# THYROXINE-LIKE ACTIVITY OF CHLORO- AND BROMO- THYRONINE DERIVATIVES

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The biological activity of thyronine derivatives has been estimated by their effect on tadpole metamorphosis (1, 2), oxygen consumption (3), carbon dioxide production (1) and survival in closed vessels (4). It was found that thyronine derivatives halogenated by bromine or chlorine could have biological activity (1), but that complete absence of all halogens as in thyronine resulted in total inactivation (1, 2, 3, 5, 6, 7, 8). Iodinated phenols, such as 3,5-diiodotyrosine, have a negligible activity (1, 2, 3, 6, 7, 8, 9, 10, 11). 3,5-Diiodothyronine has a definite calorogenic effect (2, 3, 4, 7, 8, 9, 10), but 3',5'-diiodothyronine has been found inactive (12).

To obtain more information on the respective importance of the 3,5 and 3',5' positions<sup>1</sup> and on the relative efficacy of the individual halogens in the thyronine molecule, it was decided to re-examine several thyronine derivatives in which some or all of the iodine atoms were replaced by bromine or chlorine or hydrogen. The biological activity was estimated by administering these compounds for three weeks to thyroidectomized rats, criteria for biological activity being bi-weekly oxygen consumption and heart rate measurements, as well as determination of organ weights at the end of the experiment.

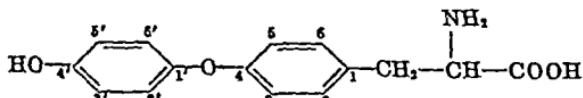
Male rats weighing from 130 to 150 grams were thyroparathyroidectomized. These animals were kept in a constant temperature room ( $28 \pm 2^\circ\text{C}$ ) illuminated from 8 a.m. to 8 p.m. Heart rate was determined electrocardiographically and expressed as beats per minute. Oxygen consumption was estimated with the help of an apparatus modified from that of Williams et al. (13). Pre-training of the animals helped to keep them quiet during the measurements. No corrections were made for temperature and pressure. The oxygen consumption results were expressed as cc. of oxygen consumed per hour per 100 square centimeters of body surface. The body surface was calculated from the body weight using Meeh's formula:

$$S = 11.2 W^{2/3}$$

where  $W$  is the body weight of the rat in grams, and  $S$  is the body surface in square centimeters.

In preliminary experiments (7, 8) 5 groups of thyroidectomized rats were set up, one group serving as control and the remaining groups receiving 3, 30, 306, 3060 micrograms of thyroxine daily for 3 weeks. The average heart rate and

<sup>1</sup>The positions in the thyronine molecule are numbered as follows:



The thyroxine molecule includes 4 iodines in the 3,5, 3',5' positions.

active material following oral or intravenous administration to nonmalarial individuals consists in determination of the antirespiratory activity per colorimetric unit of total naphthoquinone pigment extracted from plasma samples drawn at varying periods of time.

2. Compounds with hydrocarbon side chains terminating in a ring are rapidly metabolized to products hydroxylated in this ring. The metabolites retain 10-60 per cent of the original activity, often persist in the blood for long periods, and in some instances may exert a therapeutic effect.

3. A synthetic compound having a hydroxyl group in a large hydrocarbon side chain shows improved resistance to metabolic deactivation, possesses adequate activity (i.v.), and appears to offer promise as a drug.

4. Of various animals investigated for the laboratory evaluation of new compounds, the mouse shows the closest parallelism to man in the retention and degradation of the naphthoquinones. Drug degradation *in vitro* was accomplished in a mouse liver homogenate.

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weights are recorded in table I. The per cent increase over controls was calculated from the oxygen consumption and heart rate averages of table I and reported in table II where the compounds are listed in decreasing order of activity

TABLE I

*Action of thyronine derivatives on heart rate and organ morphology*

Each line in the table corresponds to a group of 6 thyroidectomized rats.

	AVERAGE HEART RATE	AVERAGE ORGAN WEIGHTS (MG.M.)		
		Adrenal	Heart	Kidney
I. thyroidectomized controls	322 ±12	28 ±2.3	557 ±35	1323 ±40
3,5-dichlorothyronine	312 ±11	31 ±1.2	623 ±48	1405 ±43
3,5 diodo-3',5'-dichlorothyro-	400 ±18	41 ±2.4	659 ±45	1595 ±93
nine . . . . .	331 ±18	32 ±2.6	614 ±27	1393 ±58
II. thyroidectomized controls	303 ±12	30 ±1.9	689 ±45	1110 ±47
3,5,3',5'-tetraiodothyronine	357 ±12	41 ±9.0	780 ±33	1471 ±47
(thyroxine, 15γ)	415 ±18	41 ±2.0	861 ±52	1651 ±58
3,5,3',5'-tetrabromothyronine .	316 ±19	26 ±1.7	669 ±40	1306 ±37
III thyroidectomized controls	356 ±11	31 ±1.9	745 ±82	1422 ±74
3,5-duodothyronine . . . . .	390 ±16	38 ±1.1	762 ±36	1561 ±20
3,5-dibromo-3',5'-diiodothyro-	484 ±46	44 ±0.3	719 ±47	1763 ±71
nine				

TABLE II

*Percentage increase of the oxygen consumption (cc./hr./100 sq. cm.) over that of thyroidectomized controls after administration of bromo- and chloro-thyronine derivatives*

1. thyroxine.	..	+184.0
2. 3,5-dibromo-3',5'-diiodothyronine		+132.5
3. 3,5-diodo-3',5'-dibromothyronine		+54.5
4. 3,5-duodo-3',5'-dichlorothyronine.		+50.0
5. 3,5,3',5'-tetrabromothyronine		+44.2
6 3,5-duodothyronine		+25.5
7. 3,5-dichloro-3',5'-diiodothyronine ..		+13.6
8. 3,5-dichlorothyronine . . . . .		+6.4
9. thyronine.	.. . . . .	—

and numbered. The calculated values were then used as ordinates for plotting along the straight line graphs of figure 1. The order of the efficiency with which these thyronine derivatives can serve as thyroxine substitutes was almost identical whichever criterion was chosen (table II, fig. 1).

oxygen consumption of each group measured during the last week were expressed as per cent increase over controls; these values were plotted as ordinates against the logarithm to base ten of the dose of thyroxine taken as abscissae (fig. 1). The line obtained for oxygen consumption values was steeper than that obtained

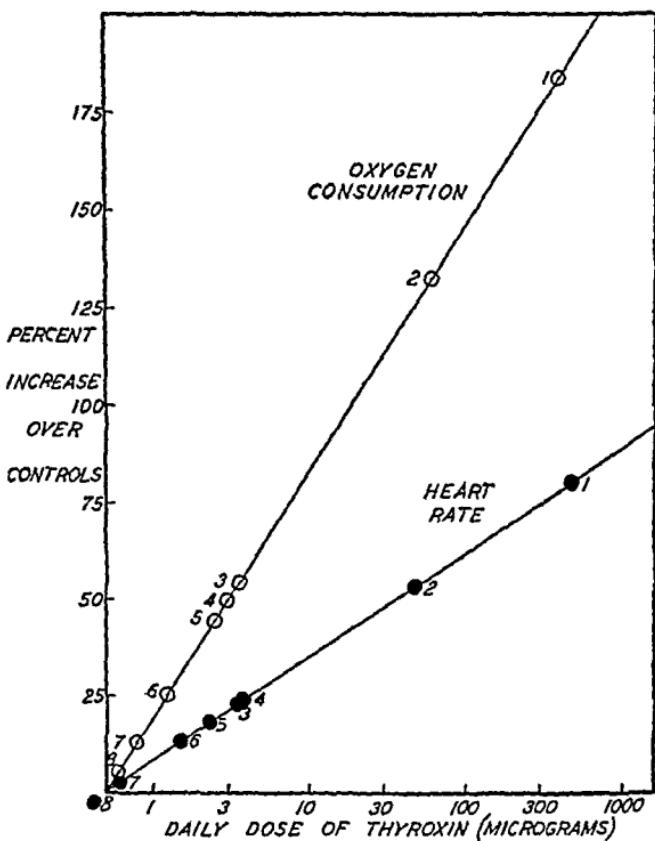


FIG. 1. The straight lines which relate the dose of thyroxine to its effect on oxygen consumption and on heart rate, were obtained in preliminary experiments with graded doses of thyroxine.

The per cent increase over controls obtained by measuring oxygen consumption in animals receiving a thyronine derivative were placed on the lines as ordinates. Each thyronine derivative on the line may be identified by its number, which is the same as used in table II. By reading off the value of the abscissa, an estimate of the activity as micrograms of thyroxine may be obtained.

for heart rates. Therefore, oxygen consumption may be a more sensitive test of thyroxine's action.

The thyronine derivatives were then assayed in the same way as the graded doses of thyroxine. A dose of 0.25 mgm. of each test substance in 0.1 cc. distilled water was administered subcutaneously twice daily for 20 to 21 days following thyroparathyroidectomy. Thus, the animals received 0.5 mgm. daily, with the exception of the rats given 5,5-diiodothyronine which received only 0.383 mgm. daily. The oxygen consumption and heart rates, as well as organ

weights are recorded in table I. The per cent increase over controls was calculated from the oxygen consumption and heart rate averages of table I and reported in table II where the compounds are listed in decreasing order of activity

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Thyronine displayed no activity. This result confirmed that the elimination of halogens from the molecule of thyroxine suppressed its characteristic biological effect.

On the other hand, iodine was not indispensable for biological activity since 3,5,3',5'-tetrabromothyronine had a definite effect (tables I and II). This was in confirmation of previous work (1, 3).

The replacement of the 3,5 bromines in the last compound by two iodines, giving the 3,5-diido-3',5'-dibromothyronine, did not significantly raise biological activity over that of 3,5,3',5'-tetrabromothyronine. Conversely, the presence of two iodines in the 3',5' positions with bromines in 3,5 produced a highly active compound, the 3,5-dibromo-3',5'-diiodothyronine, which could be roughly estimated to have one-eighth the activity of thyroxine. In other words, out of the two bromoiodo compounds, only the one with the iodine in the 3',5' positions was highly active. These results were not in agreement with those of Abderhalden and Wertheimer (1) who found the relative activities of the above two compounds were reversed.

At any rate, with either iodine or bromine in the 3',5' positions, iodine in 3,5 was more effective than bromine (as shown by the comparison of No. 1 with No. 2 and No. 3 with No. 5 in table II) and in turn, bromine in 3,5 was more effective than chlorine (as shown by comparison of No. 2 with No. 7). While the presence of iodines in the 3',5' positions may yield compounds of great activity (Nos. 1 and 2), such iodines did not guarantee high activity, as seen by the low efficacy of compound No. 7. Thus, the presence of bromine or iodine in 3,5 was necessary to enhance the activity of compounds with 3',5' iodines to a high level.

With regard to the importance of the 3',5' iodines, it has been found that, when these iodines were displaced to the 2',4' positions—a modification that involved a change in the position of the phenolic hydroxyl from the 4' to the 3' position—the compound thus obtained (meta-thyroxine) had no biological activity (14, 15). If the hydroxyl, however, was displaced from the 4' to the 2' positions, leaving the 3',5' iodines in place, the resulting thyroxine isomer (ortho-thyroxine) was still active, since it had retained one-twenty-fifth to one-fiftieth the activity of thyroxine (15, 16). Ortho-thyroxine and thyroxine, but not meta-thyroxine have iodines in 3',5' and also can form quinones (15, 16). These two characteristics may be important for biological activity.

In regard to the thyronine derivatives with iodines in the 3,5 positions, no highly active compound was obtained besides thyroxine; still all the compounds with iodine or bromine in this position had an appreciable effect, which was greater than that of one of the compounds with iodines in 3',5' (No. 7, table II). When iodines in the 3,5 positions were the only halogens present in the molecule, there was a further drop of activity, as shown by 3,5-diiodothyronine (No. 6). When chlorines in 3,5 were the only halogens in the molecule, there was no activity (No. 6).

#### CONCLUSION

These results emphasize the predominant role of iodine in the thyroxine molecule. However, the complete or partial replacement by bromines or the partial

replacement by chlorines reduces but does not necessarily eliminate biological activity.

When iodine is in the 3,5 positions, the halogen position in 3',5' is much more effectively filled by iodine than by either bromine or chlorine. Conversely, if one considers the compounds with iodine in the 3',5' positions, iodine again is more effective in 3,5 than bromine, which in turn is much more effective than chlorine. Therefore, for an activity comparable to that of thyroxine, iodine must be in 3',5' with either iodine or bromine in 3,5.

This work was carried out with the support of the Blanche Hutchison Fund of McGill University, and of the Banting Research Foundation. We wish to acknowledge the help from Dr. H. E. Hoff during the first part of this investigation. All the compounds were supplied by Hoffmann-LaRoche, Ltd., Montreal, through Mr. P. Blanc.

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# ENZYMIC HYDROLYSIS OF MORPHINE ESTERS

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Several years ago Wright (1) reported that there is present in the blood serum of some (but not all) rabbits an enzyme which is able to hydrolyze monoacetylmorphine (6-acetylmorphine). Atropine esterase is another enzyme found in the serum of only a portion of the rabbit population (Bernheim and Bernheim (2) and others (3, 4)). Sawin and Glick (5), in cooperation with Wright, determined the activities of the sera of a group of rabbits on monoacetylmorphine and on atropine. They stated that the sera either hydrolyzed both substrates or neither and that "the order of activity is the same when the serums are arranged as to enzyme concentration for either 'monoacetylmorphinease' or 'atropinesterase'". Wright (6), however, was of the opinion that the two substrates were attacked by different enzymes, since atropinesterase is not found in the livers of rabbits which lack this activity in their sera, whereas monoacetylmorphine esterase is present in the livers of rabbits, whether or not the enzyme is present in the serum.

Benzoylcholine esterase is another enzyme which does not occur in the sera of all rabbits (7). It was demonstrated that, although there is a parallelism between the activities of various rabbit sera on benzoylcholine and atropine, the two substrates are attacked by different enzymes. Although it was considered possible that only two enzymes might be involved in the hydrolysis of monoacetylmorphine, atropine, and benzoylcholine, the results of this study indicate that three separate enzymes are necessary. This report also includes some experiments on the enzyme responsible for the hydrolysis of the 3-acetyl group of heroin.

**METHODS.** Both heparinized blood plasma and serum have been used. Red cells were prepared by washing the cells repeatedly with saline. Weighed portions of washed liver were homogenized in a Waring blender with four times the weight in volumes of 0.025 M sodium bicarbonate. Determinations were made with the conventional Warburg constant volume manometric method. The medium was 0.025 Molar sodium bicarbonate in equilibrium with 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub>. Three cc. of substrate were placed in the main compartment of the vessel and 0.2 cc. of the enzyme preparation in the side-bulb. The concentrations of substrates in the final reaction mixtures were: monoacetylmorphine hydrochloride ·3H<sub>2</sub>O,  $2.25 \times 10^{-3}$  M.; atropine sulfate,  $2.7 \times 10^{-3}$  M.; homatropine sulfate,  $7.5 \times 10^{-3}$  M.; benzoylcholine chloride,  $5.8 \times 10^{-3}$ ; diacetylmorphine hydrochloride,  $2.32 \times 10^{-3}$  M.; methylbutyrate,  $3.3 \times 10^{-2}$  M.; tributyrin,  $1.29 \times 10^{-2}$  M. (suspension); acetyl-salicylic acid,  $1.88 \times 10^{-2}$  M.

We are indebted to Dr. Lyndon F. Small of the National Institute of Health for the morphine esters used in this study and to Dr. Elmer L. Sevringshaus, Hoffman-LaRoche, Inc., Nutley, New Jersey, for benzoylcholine chloride and neostigmine.

RESULTS. A group of plasmas taken from 17 rabbits was tested. Seven showed negligible hydrolytic activity on monoacetylmorphine, atropine and benzoylcholine. If the rate of hydrolysis of benzoylcholine of the active plasmas is arbitrarily called 100, the activities on monoacetylmorphine and atropine are 130 and 70, respectively. The ratios of these activities of various plasmas are quite constant. The first group of columns of figure 1 summarizes the results.

The monoacetylmorphine esterase of rabbit's blood is found only in the plasma since washed hemolyzed red cells separated from active plasma do not hydrolyze monoacetylmorphine. Wright (6) found that this ester is not attacked by rat serum. Our results indicate that human, dog, sheep and guinea pig sera also are devoid of this activity.

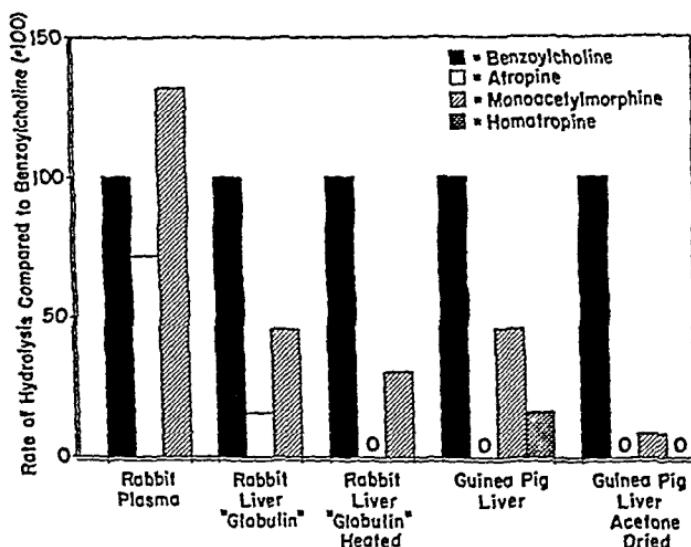


FIG. 1. THE RATES OF HYDROLYSIS OF ATROPINE, MONOACETYLMORPHINE, AND HOMATROPINE RELATIVE TO BENZOYLCHOLINE HYDROLYSIS IN SOLUTIONS OF ENZYMES PREPARED FROM TISSUES OF RABBITS AND GUINEA PIGS

THE EFFECTS OF INHIBITORS ON THREE ESTERASE ACTIVITIES. Both physostigmine and neostigmine at  $1 \times 10^{-4}$  M. are inhibitors of the esterases. The sensitivities of the enzymes to these inhibitors differ greatly. Monoacetylmorphine hydrolysis is most sensitive and atropine esterase least.

At 0.02 M. concentration, sodium fluoride shows a different order of action on the three activities. Benzoylcholine esterase is inhibited most and atropine esterase is unaffected. Tetraethylpyrophosphate (TEPP) is an inhibitor of all three activities. The sensitivities of the three activities are identical. This action of TEPP is illustrated in figure 2.

ATTEMPTS TO SEPARATE ENZYMIC ACTIVITIES IN VIVO. Tests were performed on rabbits with active sera to determine whether serum activities on the 3 substrates would reappear at the same rate after inactivating the enzymes with

TEPP. These experiments demonstrated that all 3 activities recovered at the same rate, but at a rate slightly less than the rate of renewal of acetylcholine hydrolytic activity. The recovery of the latter activity compared favorably with similar experiments reported by Mazur and Bodansky (8), who injected diisopropylfluorophosphate as the esterase inhibitor.

Further attempts to depress differentially the several enzymes of rabbit plasma with carbon tetrachloride, administered in order to damage the liver, were unsuccessful. When 0.5 cc. of  $\text{CCl}_4$  was administered intraperitoneally on alternate days, the 3 activities were reduced to 50 per cent of the normal on the fourth day of the treatment. At this time the esterases acting on acetylcholine, methylbutyrate and tributyryl were at the pre-injection level or above.

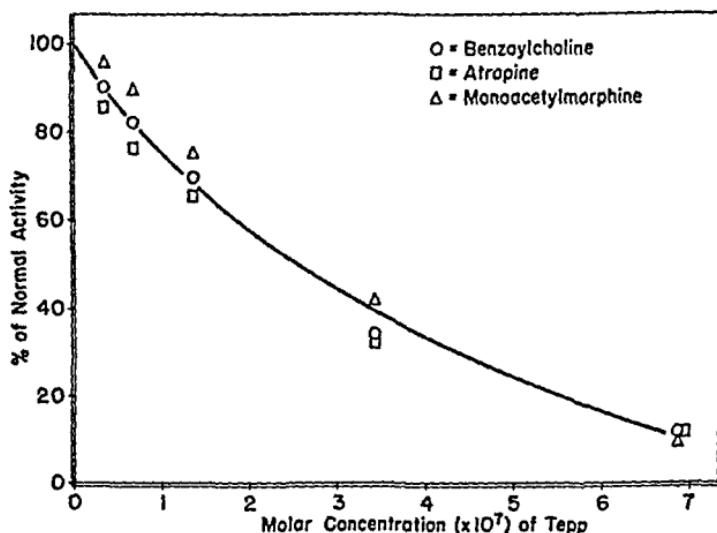


FIG. 2. INHIBITION BY TETRAETHYLPYROPHOSPHATE (TEPP) OF THE HYDROLYSIS OF BENZOYLCHOLINE, ATROPINE, AND MONOACETYLMORPHINE BY RABBIT PLASMA

**PHYSICAL SEPARATION OF THE ENZYMES.** The 3 esterase activities of the rabbit's plasma and liver all appear in the fraction precipitated by half-saturated ammonium sulfate. The liver "globulin" enzymes were found to be present in different relative amounts from those of the plasma. A comparison of the second group of columns of figure 1 with the columns representing the plasma activities illustrates this difference.

When the liver "globulin" was incubated at 48°C. for one hour, the atropine esterase was completely inactivated, monoacetylmorphine hydrolysis was reduced 50 per cent and benzoylcholine esterase was diminished 30 per cent. The relative activities of this preparation are represented by the third group of columns of figure 1. These results show a definite separation of atropine esterase from the other two activities in the same preparation.

The liver of the guinea pig contains benzoylcholine and monoacetylmorphine esterases, but this tissue hydrolyzes atropine to a negligible extent although

homatropine is readily attacked. A dry powder of guinea pig liver was prepared by dropping the liver homogenate into ten times its volume of acetone, filtering by suction and twice resuspending the powder in acetone and filtering. The dry powder had a much different relative activity on monoacetylmorphine and benzoylcholine than the original homogenate. The relative activities on the three principle substrates are shown in the fourth and fifth groups of columns of figure 1. The relative actions of these preparations on homatropine are included for comparison.

Although the enzymes of the liver were separated quite effectively by differences in sensitivity to inactivation at 48°C. or drying with acetone, plasma enzymes were not easily separated in these ways. Neither heat nor acetone drying, nor a combination of both treatments, demonstrated differences in sensitivity of the three activities of the plasma.

THE HYDROLYSIS OF THE 3-ACETYL GROUP OF DIACETYLMORPHINE (HEROIN). Wright (1) concluded that cholinesterases are not the enzymes responsible for the hydrolysis. Since recent evidence (9) indicates that there are at least two enzymes active in hydrolyzing acetylcholine, further experiments were needed to clarify this point. The plasma and liver of the guinea pig present a clear confirmation of Wright's previous studies. The plasma of this species hydrolyzes acetylcholine rapidly and acetyl- $\beta$ -methyl-choline slowly and its liver has little activity on either substrate (7, 10). The hydrolysis of diacetylmorphine, on the other hand, is rapid in both tissues with far greater activity in the liver. Some studies have been done to determine whether some known enzyme is responsible for the removal of the 3-acetyl, or phenolic acetyl group of heroin.

Smith *et al.* (11) described an enzyme which attacks acetylsalicylic acid. We found that acetylsalicylic acid is hydrolyzed rapidly by guinea pig plasma, but only slowly by plasmas of man, dog, and rabbit. Plasmas of rabbits hydrolyze this substrate at approximately the same rate whether they possess monoacetylmorphine esterase activity or not. Heroin, on the other hand, is hydrolyzed rapidly by both rabbit and guinea pig plasmas, slowly by human and not at all by dog plasmas. Thus, we must conclude that heroin is not hydrolyzed by the same enzyme that attacks acetylsalicylic acid. The above authors considered the acetylsalicylic acid hydrolysis a function of the liver esterase which attacks ethylbutyrate. Our studies of the hydrolysis of aspirin by several animal plasmas are at variance with this opinion. Of the four species studied, both the rabbit and the guinea pig had high activity on methylbutyrate. As stated above, however, only guinea pig plasma is very active in splitting acetylsalicylic acid.

The relative activities of samples of human, dog, rabbit and guinea pig sera on tributyrin, methylbutyrate and heroin made it appear possible that heroin is hydrolyzed by the plasma tributyrinase. If we accept the results of Mendel and Rudney (9), who found that purified "pseudo-cholinesterase" attacks tributyrin at one-third of the rate at which it hydrolyses acetylcholine, the tributyrin hydrolysis by dog sera is due entirely to the above enzyme. This substrate is split by tributyrinase which occurs in low concentration in human serum and in large amounts in the sera of rabbits and of guinea pigs (unpub-

lished data). The enzymic hydrolysis of heroin by horse serum has been reported by Massart and Dufait (12). This also can be attributed to tributyrinase which is present in fairly high concentration in horse serum. Tributyrinase is found in red cells (9) and it was found that hemolyzed red cells of the rabbit hydrolyze diacetylmorphine.

Some experiments with rabbit and guinea pig sera and livers in which mixtures of substrates were employed definitely eliminated the methylbutyrate esterase and made it likely that the tributyrin esterase was the responsible enzyme. It is quite evident from figure 3 that summation of activities is complete when heroin and methylbutyrate are the substrates. There is either lack of summation or possibly inhibition when tributyrin is combined with heroin.

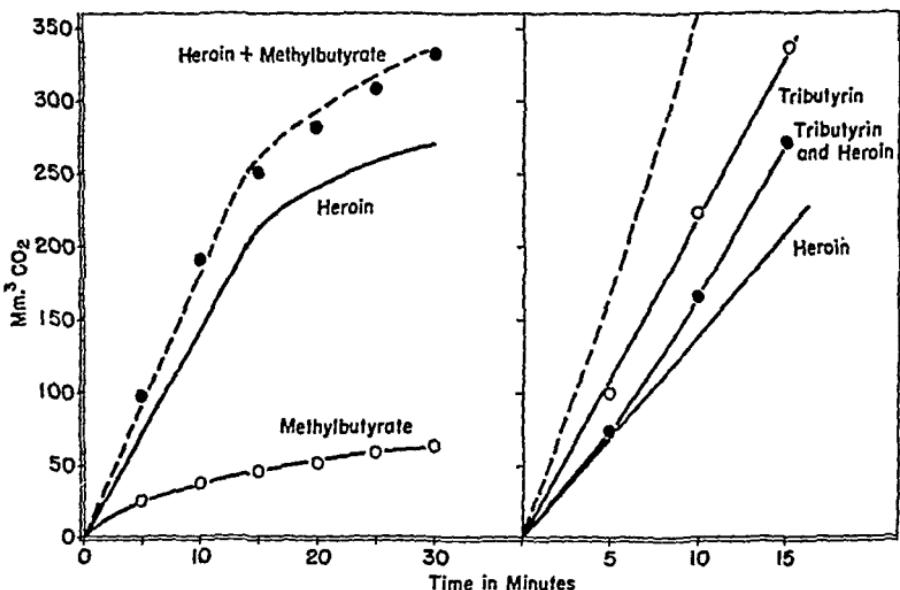


FIG. 3. THE HYDROLYSIS OF INDIVIDUAL AND MIXTURES OF SUBSTRATES BY RABBIT PLASMA

The broken lines indicate the hydrolysis expected in the presence of mixtures of substrates if the hydrolysis of each substrate were independent of the other.

Thus, we may conclude that heroin can be hydrolyzed in the following manner. The phenolic acetyl group may be removed by tributyrinase or possibly other simple esterases. The alcoholic acetyl radical is removed by an enzyme which, for lack of knowledge of its physiological substrate, may be called monoacetylmorphine esterase.

#### SUMMARY

1. The activities of the plasma and liver of the rabbit on monoacetylmorphine, benzoylcholine and atropine are due to three separate enzymes.
2. The above conclusion is based on the following observations: the relative activities on the three substrates of plasma as compared with liver; the distribu-

tion of the various enzymes in the tissues and bloods of other animals; and the differences in sensitivity of the various activities to esterase inhibitors, to heat and to drying with acetone.

3. The hydrolysis of the 3-acetyl group of heroin appears to be a function of tributyrylase, but not of the enzymes which hydrolyze methylbutyrate, acetyl-choline, or acetylsalicylic acid.

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# FURTHER STUDIES ON THE ANTIFIBRILLATORY ACTION OF CORONARY DILATOR DRUGS<sup>1</sup>

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In earlier publications from this laboratory (1, 2), it was shown that atabrine and papaverine can protect the heart against ventricular premature contractions and fibrillation, following injections of epinephrine during chloroform inhalation in dogs under pentobarbital anesthesia. It was also shown in these experiments that the intracardiac injection of papaverine in conjunction with cardiac massage can stop chloroform-epinephrine ventricular fibrillation previously induced under similar conditions, and restore a normal coordinated heart beat. Since both atabrine (3) and papaverine (4, 5) are potent coronary dilator agents, it was suggested that their antifibrillatory effects were due to this action.

In view of these observations it was of interest to test the effects of various other types of coronary dilator drugs against chloroform-epinephrine ventricular premature contractions and fibrillation. The object of this paper is to present the results obtained with ephedrine, amyl nitrite, sodium nitrite, and nitroglycerine.

The effects of ephedrine on chloroform-epinephrine ventricular fibrillation have not previously been tested, probably because as is well known, ephedrine itself can induce ventricular premature contractions. It has however, been repeatedly noted that ephedrine, unlike epinephrine, never induces ventricular fibrillation during chloroform administration.

Dautrebande and Charlier (6, 7) have studied the effects of amyl nitrite and sodium nitrite in both benzol-adrenaline, and chloroform-adrenaline syncope, and have reported that both of these agents can protect the circulation against these types of collapse. The electrocardiographic changes associated with these effects were not studied by these workers, and it was suggested that the favorable effects of the nitrites are due to their peripheral vasodilator actions causing a fall in blood pressure which prevents the adrenaline pressor response and hence the initiating factor in adrenaline fibrillation.

In view of this suggestion and in view of the extremely high blood pressure levels observed without fibrillation during the course of the experiments to be described with ephedrine, it was also of interest to study the influence of chloroform inhalation upon the circulation, while the blood pressure was being maintained at a high level by the continuous intravenous injection of epinephrine itself.

<sup>1</sup> Presented in part at the III Interamerican Cardiological Congress, Chicago, June 15th, 1948.

**METHODS.** Dogs anesthetized with sodium pentobarbital were used. Artificial respiration was maintained throughout by means of a Starling pump attached to a tracheal cannula.

Chloroform was administered as desired by connecting the chloroform bottle with the inlet of the artificial respiration circuit close to the tracheal cannula, as previously described (1). After 5 minutes of chloroform the effects of intravenous injections of either a small dose (0.002 mgm. per kgm.) or a large dose (0.02 mgm. per kgm.) of epinephrine were tested. The coronary dilator agent to be studied—ephedrine (0.5 to 5 mgm. per kgm.), amyl nitrite (15 to 30 secs. inhalation), sodium nitrite (10 to 20 mgm. per kgm.) or nitroglycerine (0.05 to 0.5 mgm. per kgm.)—was administered 1 to 2 minutes prior to the epinephrine.

Blood pressure was recorded directly from a common carotid artery and electrocardiograms (Lead II) taken at frequent intervals during the course of all experiments.

In some experiments the vagus nerves were cut, either at the beginning or during the course of the experiment.

TABLE 1

*Effects of ephedrine on occurrence of ventricular premature beats and fibrillation following injection of epinephrine during chloroform*

EXP. NO.	WEIGHT OF DOG	DOSE OF EPHEDRINE	DOSE OF EPINEPHRINE	RESULTS
	kgm.	mgm. per kgm.	mgm. per kgm.	
1	6.5	2.5	0.002	Premature beats, no fibrillation
2	9.0	2.5	0.02	Premature beats, no fibrillation
3	9.0	2.5	0.002	No premature beats, no fibrillation
4	13.2	5.0	0.02	Premature beats, no fibrillation
5	5.1*	0.5	0.02	Premature beats, no fibrillation
6	5.0*	1.0	0.002	No premature beats, no fibrillation
7	5.0*	1.0	0.02	Premature beats, no fibrillation
8	5.0*	2.5	0.02	Premature beats, no fibrillation
9	34.8*	5.0	0.02	Fibrillation

\* Vagotomized.

All injections were made into an exposed femoral vein. Amyl nitrite was administered by inhalation in similar fashion to chloroform—the container of amyl nitrite replacing the chloroform bottle in the artificial respiration circuit for 10 to 30 seconds, as needed.

**RESULTS.** 1. *Effects of ephedrine.* The effect of a previous injection of ephedrine upon the chloroform-epinephrine response was tested in nine experiments. The results obtained are summarized in table 1.

Figures, 1, 2 and 3 show some examples of these results. Thus, from fig. 1, it may be seen that during a prolonged pressor response to ephedrine during chloroform, injection of a small dose of epinephrine induced a further pressor response associated with a sinus tachycardia and progressive T-wave depression but no ventricular premature beats (records nos. 5a and 5b). Five minutes later injection of a large dose of epinephrine during similar chloroform administration again led to an irregular pressor response associated with several ventricular premature contractions but no fibrillation (records nos. 8a, 8b, and 9). These ventricular irregularities persisted until the vagi were cut (record no. 10).

Figure 2, which is a continuation of the previous experiment, shows the effects



FIG. 1. Dog, male, 9.0 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Blood pressure tracing and electrocardiograms (Lead II) taken at nos. 1 to 10—records nos. 5a and 5b, and 8a and 8b, respectively, are continuous. A—ephedrine (2.5 mgm. per kgm.). B—epinephrine (0.002 mgm. per kgm.). C—continuous.

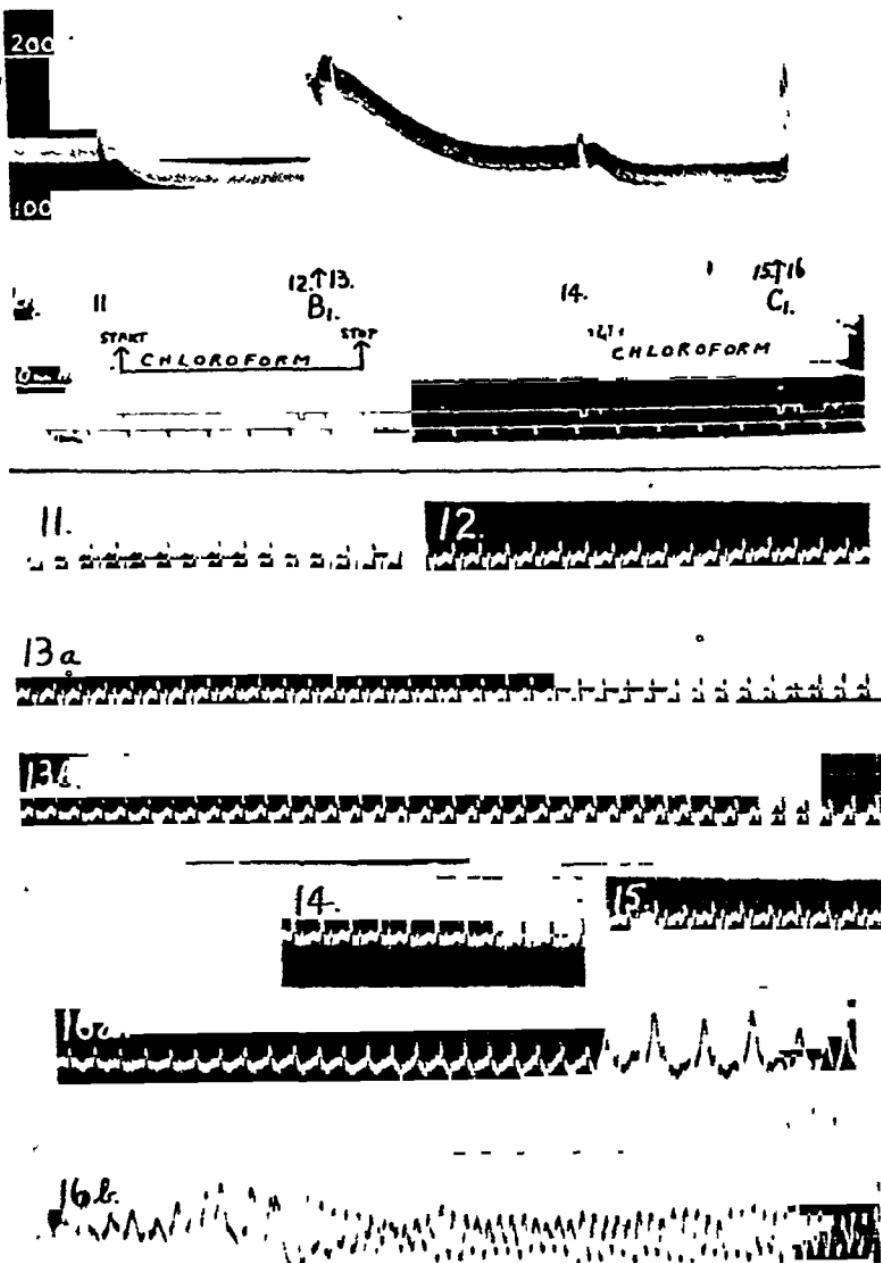


FIG 2 Continuation of experiment shown in fig 1 (after an interval of 60 minutes) Blood pressure tracing and electrocardiograms (Lead II) taken at nos 11 to 16—records nos 13a and 13b, and 16a and 16b, respectively, are continuous B<sub>1</sub>—epinephrine (0.02 mgm per kgm) C<sub>1</sub>—epinephrine (0.02 mgm per kgm)

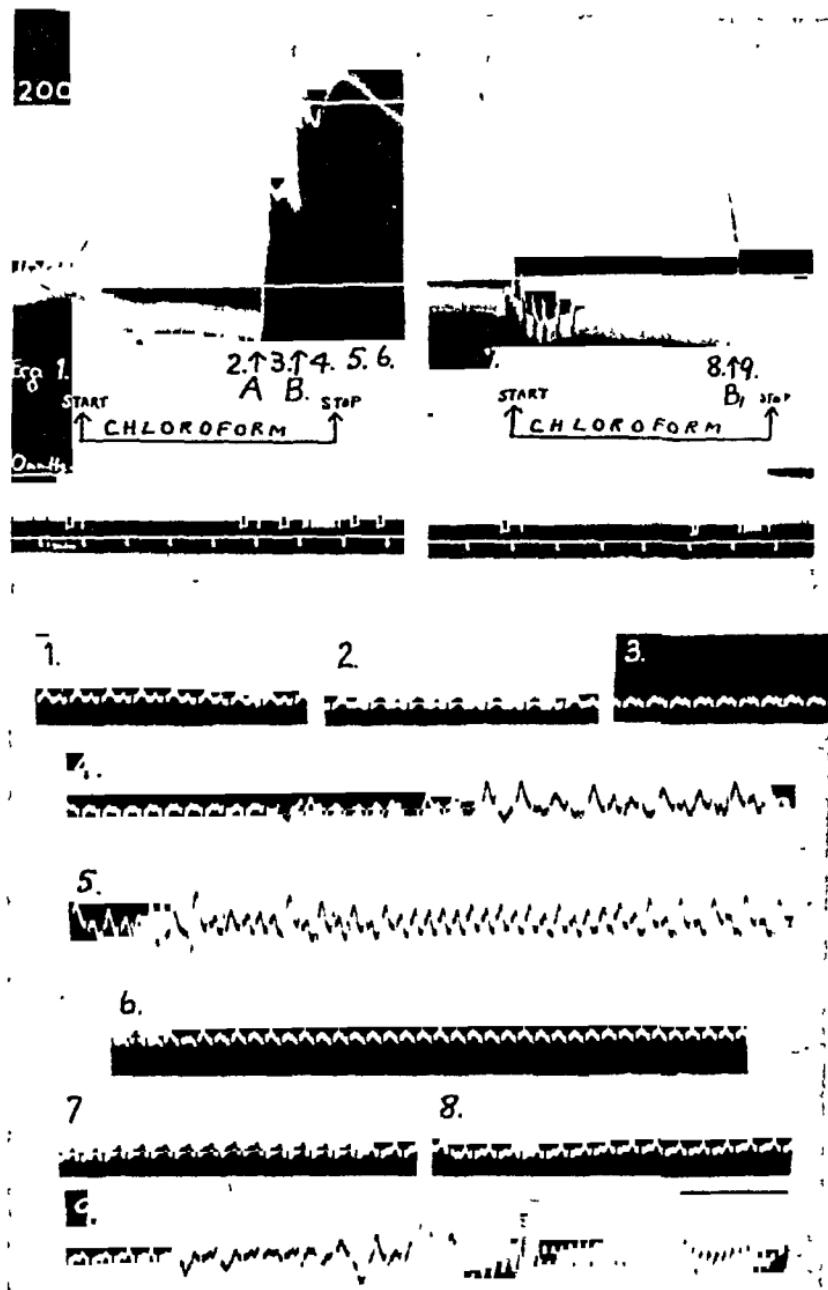


FIG. 3. Dog, female, 5.0 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Vagotomized. Blood pressure tracing and electrocardiograms (Lead II) taken at nos. 1 to 9. A—ephedrine (2.5 mgm. per kgm.). B and B<sub>1</sub>—epinephrine (0.02 mgm. per kgm.). An interval of 45 minutes elapsed between B and B<sub>1</sub>.

of similar repetitions of chloroform and epinephrine one hour later, after the blood pressure had returned to the control level. Again, the small dose of

epinephrine induced a pressor response, but still no evidence of ventricular premature contractions (records nos. 13a and 13b), as are usually observed with such a dose, showing that some effect of ephedrine was probably still present. The injection of a large dose of epinephrine however induced the usual typical ventricular fibrillation (records nos. 16a and 16b).

Figure 3 shows another example of the antifibrillatory action of ephedrine. In this experiment, following the epinephrine there was an irregular pressor

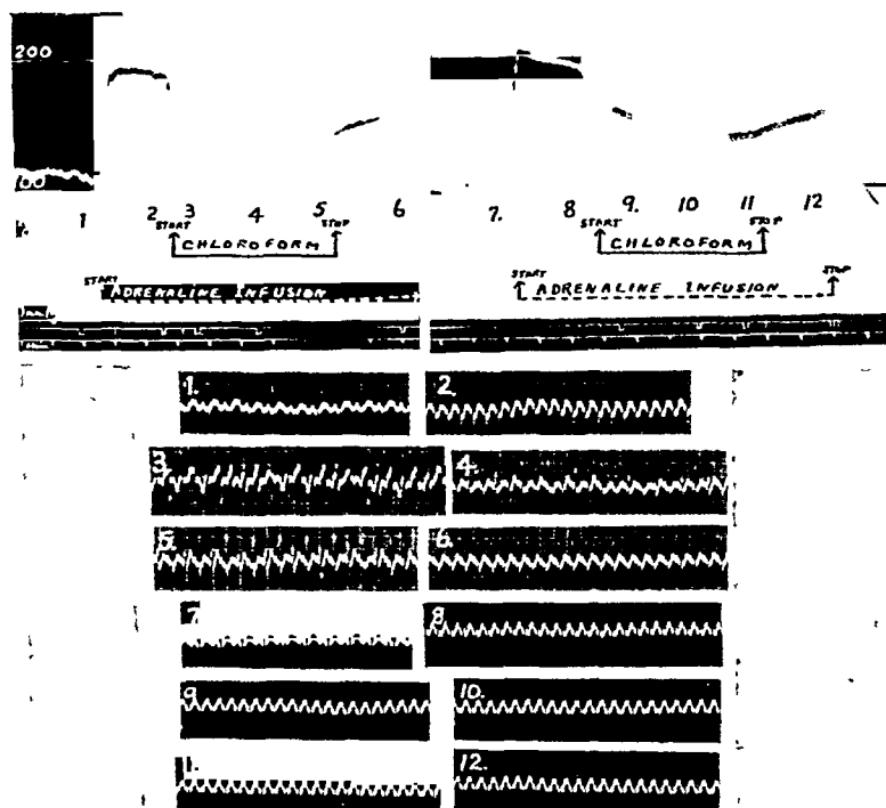


FIG. 4: Dog, male, 4.5 kgm. Sodium pentobarbital... Artificial respiration Vagotomized. Blood pressure tracings and ... Lead II) taken at nos. 1 to 12. An interval of 30 minutes elapsed between nos. 6 and 7. Periods of continuous epinephrine infusion are shown—a concentration of 0.02 mgm. per kgm. per min. was employed during the first infusion, and 0.04 mgm. per kgm. per min. during the second. The volume injected was 1 cc. per min.

response associated with the appearance of ventricular premature contractions (records nos. 4 and 5) but no fibrillation. Later, repetition of chloroform and epinephrine induced the usual ventricular premature contractions followed by fibrillation (record no. 9).

In some of the experiments with ephedrine it was also observed that ventricular premature contractions sometimes followed the injection of similar doses of ephedrine during chloroform. The superimposed injection of a large dose of epinephrine in such experiments however induced a good pressor response asso-

ciated with numerous premature contractions but no fibrillation. Furthermore, in none of the experiments with chloroform and ephedrine alone did fibrillation occur.

It is clear from the above data that ephedrine in some way or other can protect the heart against chloroform-epinephrine ventricular fibrillation, although it can induce ventricular premature contractions itself.

The extremely high blood pressure levels shown in the above experiments would also suggest that in chloroform-epinephrine ventricular fibrillation, the blood pressure rise *per se* is not the important or initiating factor in this phenomenon. Furthermore, as can be seen in fig. 4, two administrations of chloroform for 5 minute periods during prolonged continuous intravenous injections of epinephrine did not induce fibrillation, despite the fact that the blood pressure was being maintained at a high level (200 mm. of mercury) when the chloroform

TABLE 2

*Effects of amyl nitrite on occurrence of ventricular premature beats and fibrillation following injection of epinephrine during chloroform*

EXP. NO.	WEIGHT OF DOG	DURATION OF AMYL NITRITE ADMINISTRA- TION	DOSE OF EPINEPHRINE	RESULTS
1	7.9	15	0.002	No premature beats, no fibrillation
2	13.6	20	0.02	Fibrillation
3	8.7	30	0.02	No premature beats, no fibrillation
4	8.5	30	0.02	No premature beats, no fibrillation
5	10.4	30	0.02	No premature beats, no fibrillation
6	10.0*	15	0.002	No premature beats, no fibrillation
7	7.0*	20	0.02	Fibrillation
8	4.0*	30	0.02	No premature beats, no fibrillation
9	8.7*	30	0.02	No premature beats, no fibrillation
10	7.9*	30	0.02	No premature beats, no fibrillation

\* Vagotomized.

was started. During the first infusion of a dose of 0.02 mgm. of epinephrine per kgm. per min. chloroform induced some ventricular irregularities (records nos. 3, 4 and 5). During the second administration of a larger dose of epinephrine (0.04 mgm. per kgm. per min.), a similar administration of chloroform induced only a slight depressor response but no evidence of ventricular premature beats or fibrillation (records nos. 9, 10 and 11). Similar results have been observed in five other experiments. These results show clearly that chloroform-epinephrine fibrillation is related to some other effect of an epinephrine injection, rather than to the blood pressure level, and also that this effect is only an initial action of the epinephrine which is not in evidence later on.

2. *Effects of amyl nitrite, sodium nitrite and nitroglycerine.* The effects of amyl nitrite were tested in 10 different experiments and the results obtained are summarized in table 2.

Figures 5 and 6 show some examples of these effects. Thus from fig. 5, it is seen that a small dose of epinephrine injected after 15 seconds of amyl nitrite induced a good pressor response but no ventricular irregularities (record no. 6). In contrast, the same dose of epinephrine injected later in the experiment during similar chloroform administration induced ventricular fibrillation (record no. 9).

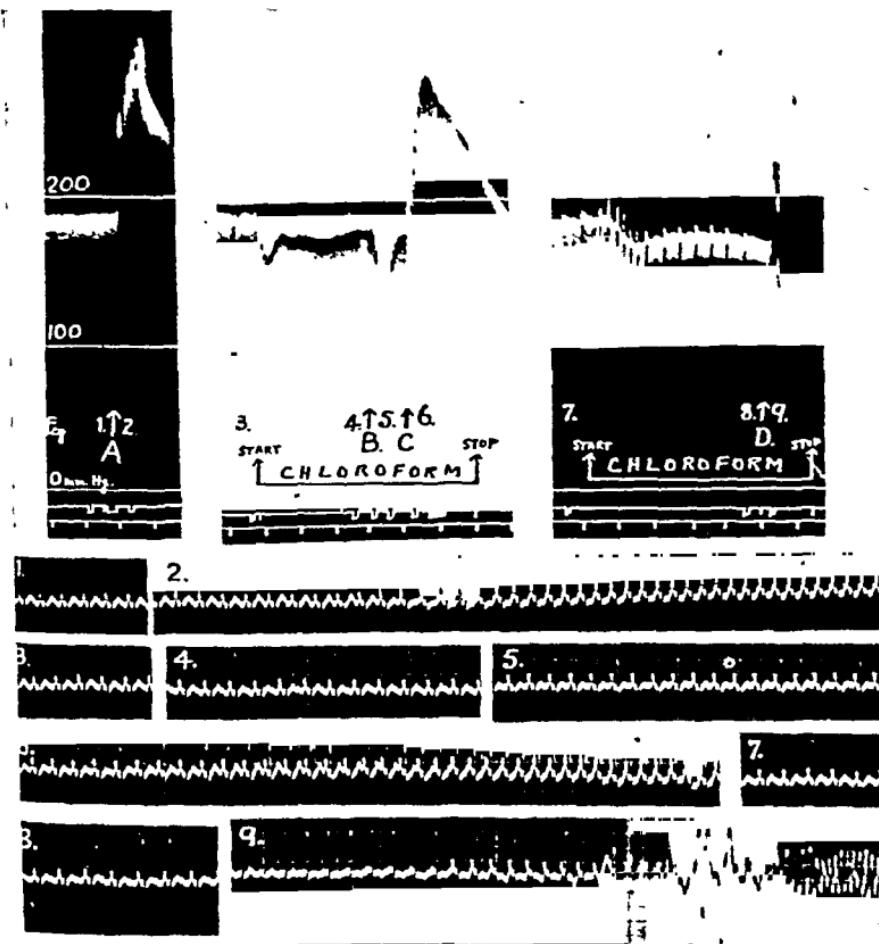


FIG. 5. Dog, female, 10.0 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Vagotomized. Blood pressure tracings and electrocardiograms (Lead II) taken at nos. 1 to 9. A, C and D—epinephrine (0.002 mgm. per kgm.). B—amyl nitrite inhalation for 15 seconds. An interval of 35 minutes elapsed between C and D, and 15 minutes between A and B.

It may also be seen that the pressor responses to epinephrine were rather similar before (A) and after (C) the amyl nitrite administration.

Figure 6 shows results obtained with large doses of epinephrine after previous amyl nitrite administration during chloroform, before and after vagotomy. In each instance there was a good pressor response but no evidence of ventricular

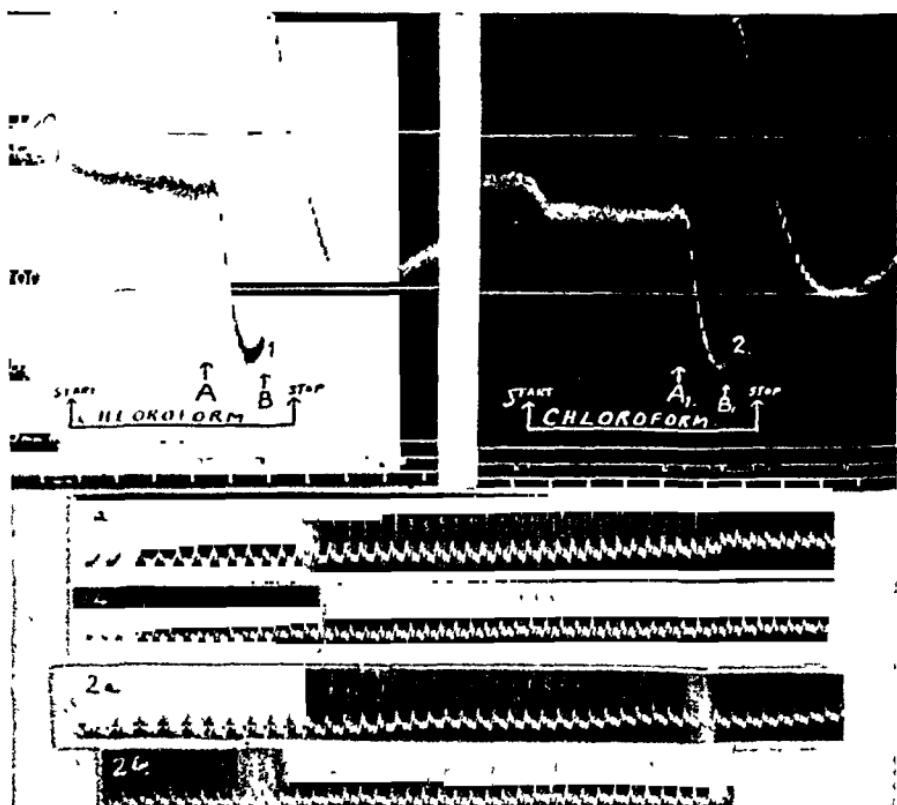


FIG. 6. Dog male 8.7 kgm. S.A. 100 mm. I.V. 10 ml. 10% glucose. Anesthesia. Artificial respiration. Blood pressure 100 mm. Hg. Heart rate 100 per minute. Record nos. 1 and 2 (1a and 1b, and 2a and 2b) taken at nos. 1 and 2 (1a and 1b, and 2a and 2b). (I) taken at nos. 1 and 2 (1a and 1b, and 2a and 2b). A—chloroform inhalation for 10 seconds. B and B<sub>1</sub>—epinephrine (0.02 mgm. per kgm.). An interval of 35 minutes elapsed between A and A<sub>1</sub>, and the vagus nerves were cut 20 minutes before A<sub>1</sub>.

TABLE 3

*Effects of sodium nitrite on occurrence of ventricular premature beats and fibrillation following injection of epinephrine during chloroform*

EXP NO	WEIGHT OF DOG	DOSE OF SODIUM NITRITE	DOSE OF EPINEPHRINE	RESULTS	
				kgm	mgm per kgm
1	8.2	10.0	0.002		No premature beats, no fibrillation
2	12.4	20.0	0.002		No premature beats, no fibrillation
3	8.2	10.0	0.02		Premature beats, no fibrillation
4	7.4	10.0	0.02		Fibrillation
5	13.8*	10.0	0.002		No premature beats, no fibrillation
6	13.8*	20.0	0.02		Premature beats, no fibrillation

\* Vagotomized

premature contractions or fibrillation (records nos. 1a and 1b, 2a and 2b). A marked sustained sinus tachycardia (300 beats per min.) occurred in both cases.

It is thus clear that amyl nitrite can effectively protect the heart against the deleterious effect of epinephrine during chloroform, and exerts this effect in doses which do not lessen the pressor responses to epinephrine. In one experiment however, when the dose of amyl nitrite was relatively large (30 seconds of

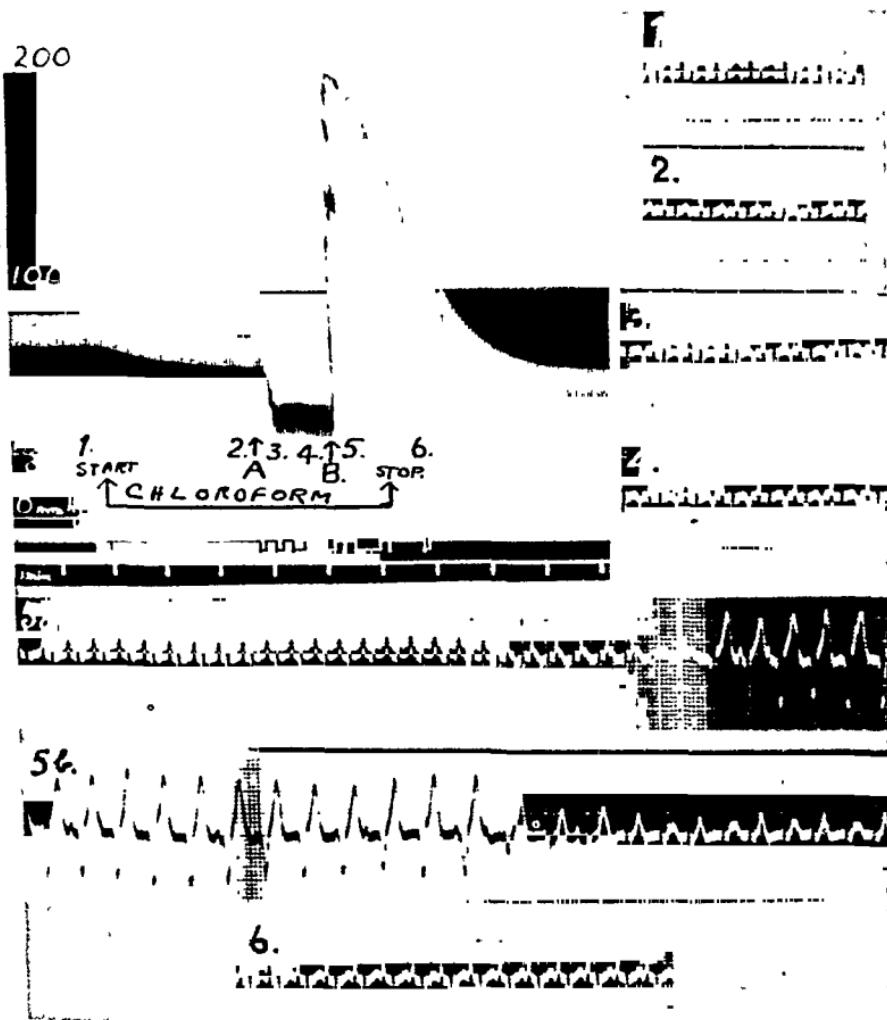


Fig. 7. Dog, male, 13.8 kgm. Sodium pentobarbital anesthesia. Artificial respiration. Vagotomized. Blood pressure tracing and electrocardiograms (Lead II) taken at nos. 1 to 6—records nos. 5a and 5b are continuous. A—sodium nitrite (20 mgm. per kgm.). B—epinephrine (0.02 mgm. per kgm.).

inhalation in a 4 kgm. dog), there was a marked depressor response, and the injection of a large dose of epinephrine then induced only a gradual restoration of the control blood pressure level, but no evidence of the usual intense pressor response as shown in fig. 6. In this experiment however, there was again no evidence of any ventricular irregularity.

Results of six experiments in which sodium nitrite was tested are summarized in table 3.

Figure 7 shows an example of one of the above experiments in which injection of a large dose of epinephrine after sodium nitrite induced a good pressor response and a transitory paroxysm of ventricular premature beats (records nos. 5a and 5b) but no fibrillation.

Even with the largest doses of sodium nitrite employed in the above experiments, the protection afforded was never as complete as in the experiments with either amyl nitrite or nitroglycerine, and transient premature contractions always occurred with large doses of epinephrine, although there was no fibrillation. It is apparent therefore that sodium nitrite is less effective than the other two agents.

Finally, in table 4 are summarized the results obtained in six experiments with nitroglycerine.

TABLE 4

*Effects of nitroglycerine on occurrence of ventricular premature beats and fibrillation following injection of epinephrine during chloroform*

EXP. NO.	WEIGHT OF DOG <i>kgm.</i>	DOSE OF NITROGLY- CERINE <i>mgm. per kgm.</i>	DOSE OF EPINEPHRINE <i>mgm. per kgm.</i>	RESULTS
1	22.0	0.05	0.002	No premature beats, no fibrillation
2	9.0	0.10	0.002	No premature beats, no fibrillation
3	9.0	0.10	0.02	Fibrillation
4	22.0*	0.05	0.02	Premature beats, no fibrillation
5	8.7*	0.5	0.02	No premature beats, no fibrillation
6	8.7*	0.5	0.02	No premature beats, no fibrillation

\* Vagotomized.

Figure 8, shows an example of one of these experiments in a vagotomized animal. This needs no further comment. A similar injection of epinephrine during chloroform later in this experiment induced typical ventricular fibrillation.

These results show that nitroglycerine can protect the heart against chloroform-epinephrine ventricular premature contractions and ventricular fibrillation, and that under such conditions, adrenaline can still induce a good pressor response. It might be added that as in the experiments with amyl nitrite, when the depressor response to nitroglycerine is very intense, the pressor response to adrenaline may also be greatly lessened.

**DISCUSSION.** The experiments presented above show definitely that ephedrine, amyl nitrite, sodium nitrite, and nitroglycerine in suitable doses can protect the heart against ventricular premature contractions and fibrillation following injections of epinephrine during chloroform inhalation. In the case of ephedrine, it is clear, however, that this effect is complicated by the tendency of

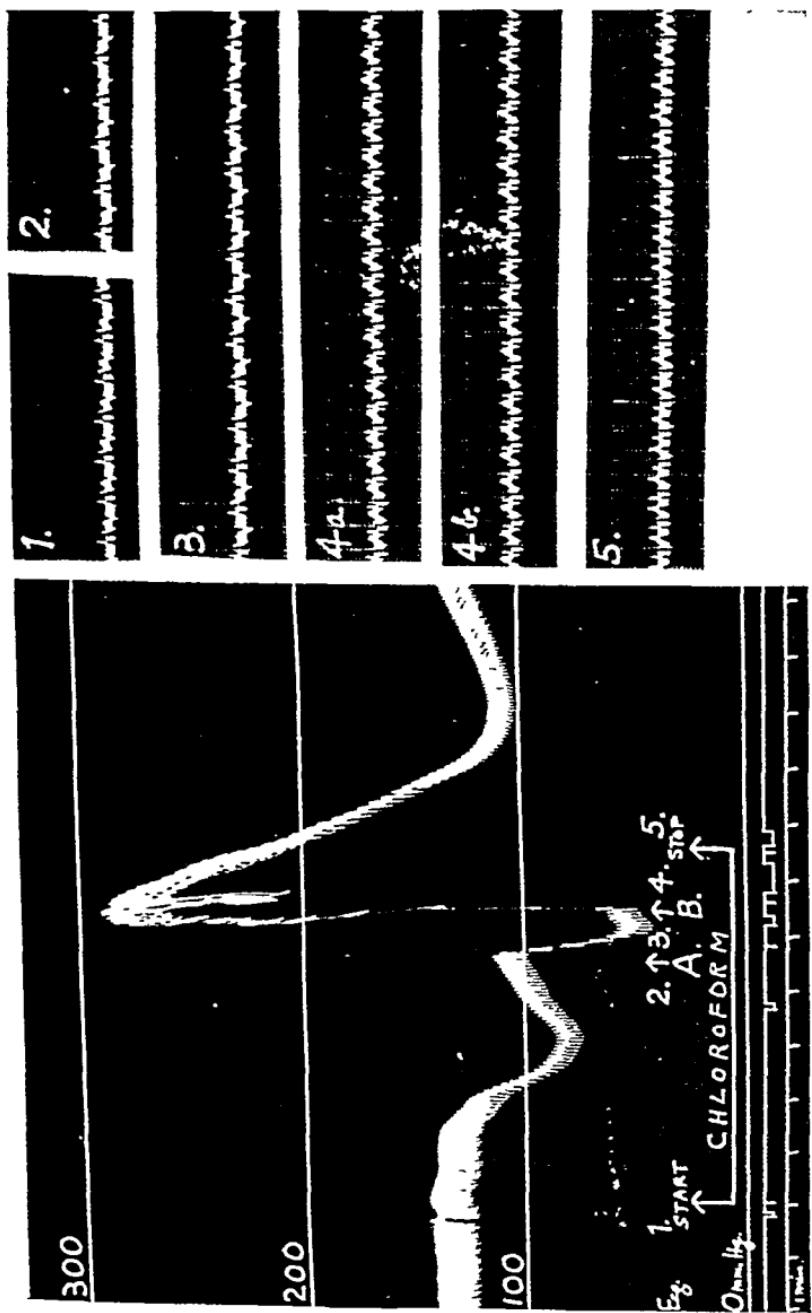


Fig. 8. Dog, male, 8.7 kgm. Sodium pentobarbital anesthetized. Artificial respiration. Vagotomized. Blood pressure tracing and electrocardiograms (Lead II) taken at nos. 1 to 5—records nos. 4a and 4b are continuous. A—nitroglycerine (0.5 mgm. per kgm.). B—epinephrine (0.02 mgm. per kgm.).

ephedrine itself to induce ventricular premature contractions. Despite this fact and despite the intense pressor responses observed with the combination of ephedrine and epinephrine, ventricular fibrillation does not ensue. Moreover, it has also been demonstrated that the inhalation of chloroform for periods of 5 minutes during continuous intravenous epinephrine injections does not induce ventricular fibrillation, although the blood pressure was being maintained at a high level when the chloroform was started. In addition, in the cases of amyl nitrite, sodium nitrite and nitroglycerine, it is seen that these agents protect against epinephrine cardiac irregularities without affecting the pressor response to epinephrine. Larger doses of these agents may however lessen the pressor response to adrenaline.

The above results do not indicate that ventricular premature contractions and fibrillation induced by epinephrine are due primarily to the abrupt pressor effect of epinephrine but rather to some other factor which is present immediately after the epinephrine injection but not in evidence during prolonged injection of epinephrine.

As in our previous studies, the results obtained on the vagotomized and on the non-vagotomized animal, do not suggest any fundamental difference in respect to the initiation of ventricular fibrillation under these different conditions. It is obvious, however, that vagotomy can abolish some ventricular premature contractions, which occur particularly when the blood pressure is exceedingly elevated. On the other hand, ventricular premature contraction and fibrillation readily occur in non-vagotomized animals with large doses of epinephrine.

In view of the fact that none of these drugs in antifibrillatory doses induces any evidence of diminished myocardial conduction judging from the electrocardiograms, and in view of the fact that all of these agents are known to dilate the coronary blood vessels, it is suggested that their antifibrillatory effects are due to this latter action.

#### SUMMARY

1. In dogs anesthetized with sodium pentobarbital and artificially respiration, administrations of ephedrine, amyl nitrite, sodium nitrite and nitroglycerine in suitable doses, can protect the heart against ventricular premature contractions and fibrillation following injections of epinephrine during chloroform inhalations. Under these conditions the pressor response to epinephrine is not prevented by the nitrites and may be added to that of ephedrine.

2. It is also shown that when the blood pressure is being maintained at a high level by continuous intravenous injections of epinephrine, similar chloroform inhalations do not induce fibrillation.

3. These results do not suggest that the pressor response to epinephrine is the initiating factor in this type of fibrillation.

4. It is suggested that the antifibrillatory effects of these agents are due to their coronary dilator actions.

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# A PHARMACOLOGIC STUDY OF ORTHO-METHOXY-beta-PHENYL-ISOPROPYL METHYLAMINE HYDROCHLORIDE, AND THIRTEEN RELATED METHOXY ANALOGUES

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In these laboratories we have studied a large series of *n*-propyl and isopropyl amines chemically similar to ephedrine. The purpose of this investigation was to obtain compounds more active than ephedrine as bronchodilators and possessing little or no pressor activity. The hydroxy and methoxy-phenyl-*n*-propylamine series was synthesized (1, 2), and the bronchodilator activity, toxicities, and pressor properties were reported (3). The conclusion reached was that the most active bronchodilator compounds, as determined by perfusion of the isolated rabbit's lung according to the method of Sollmann and von Oettingen (4), generally were methoxy derivatives with low pressor activity. This lack of parallelism of bronchodilation and pressor activity offered further evidence, therefore, that amines of this type cannot be adequately evaluated by pressor ratios alone.

Although several of these  $\beta$ -methoxyphenyl-*n*-propylamines possessed comparatively high bronchodilator activity, when compared with ephedrine in the lung perfusion experiments, they were relatively less effective than ephedrine when tested orally in human cases of asthma. Such lack of oral efficacy can be explained, in the light of the findings of Beyer (5), on the basis that the tissue enzyme, amine oxidase, rapidly oxidizes the normal propyl amines, those having the amino group on the terminal carbon atom of the side chain. *In vitro*, enzymic oxidative deamination studies in this laboratory showed, as Beyer found with ephedrine, that the methoxyphenyl isopropylamines, in contrast with the *n*-propylamines, were not oxidatively deaminated. A series comprising primary, secondary, and tertiary methoxyphenyl isopropylamines was therefore synthesized (6, 7).

Some of these compounds were included in a previous brief summary of pharmacologic data obtained with a large number of compounds of this series (8). The enhancement of bronchodilator effect, according to earlier observations, generally was obtained by employing methoxyphenyl rather than hydroxyphenyl groups, by the use of the isopropyl rather than the *n*-propyl groups, and by the addition of a methyl group on the amino group to form a secondary amine. Further work has substantiated these preliminary observations and it can be concluded, thus far, that the compound ortho-methoxy- $\beta$ -phenylisopropyl methylamine hydrochloride, for which the name 'Orthoxine' has been chosen, possesses in a very high degree a specific bronchodilator activity with little or no pressor activity. More extensive studies were carried out with 'Orthoxine', in which it was compared directly with ephedrine. Its specific

effect in counteracting spasms was further shown by its greater potency than ephedrine in relieving intestinal smooth-muscle spasms as well as bronchial-muscle spasms induced by the constrictor agents pilocarpine, histamine, acetyl-choline and barium cations.

**METHODS.** The pharmacologic studies made on this series of methoxy isopropylamines include acute toxicities, blood pressure determinations, and bronchiolar actions. Details of these preliminary evaluating methods were previously outlined in this journal (3). The toxicity data summarized in table 1 are based on a total of 293 rats. Each rat was used only once. After preliminary orientation tests, not less than three groups of five rats each were used in the range of the  $L.D_{.50} \pm 10$  mgm. per kgm.

In addition, 'Orthoxine' has been studied in more detail and compared directly to ephedrine sulfate. Smooth-muscle tests have been carried out (method of Magnus) using the isolated ileum of the rabbit and jejunum of the guinea pig. The effect of this substance on isolated rabbit uterine strips also was determined.

The bronchodilator activity of 'Orthoxine' was evaluated in comparison with ephedrine using the three bronchoconstrictor agents pilocarpine, histamine, and acetylcholine.

A comparison of the rates of oxidative deamination of 'Orthoxine', ephedrine, and several *n*-propylamines by amine oxidase was made by tests *in vitro* employing the Warburg apparatus.

Blood pressure studies were done on adult male dogs anesthetized with morphine-chlortone orally and sodium phenobarbital intraperitoneally.

Heart rate and respiratory rate determinations were made on unanesthetized adult male dogs.

Acute toxicities were carried out intravenously, orally, and subcutaneously in both rats and rabbits. The toxicity data summarized in table 3 are based on a total of 247 rats and 93 rabbits. Each animal was used only once. After preliminary orientation tests not less than 3 groups of 5 rats and 3 rabbits each were used in the range of the  $L.D_{.50}$ . Intravenously in rats and rabbits the range was  $L.D_{.50} \pm 10$  mgm. per kgm. Subcutaneously in rats and rabbits the range was  $L.D_{.50} \pm 25$  mgm. per kgm. Orally in rats and rabbits the range was  $L.D_{.50} \pm 50$  mgm. per kgm. All the toxicity data in table 3 are calculated using the method of Dragstedt.

Chronic toxicity studies involving daily doses for 21 consecutive days were done using 80 rats, 30 rabbits, and 15 guinea pigs. Each of these species was administered the test substances subcutaneously, while only rats and rabbits were administered the drugs orally by catheter. Immediately following the termination of the chronic toxicity study, all the animals involved were subjected to autopsy, and tissues were preserved for pathologic examination.

**RESULTS.** Table 1 outlines the preliminary pharmacological results obtained with this isopropyl amine series. Primary, secondary, and tertiary amines were studied with substitutions on both the amino-nitrogen and the phenyl-nucleus. Only monomethoxy substitutions in the ortho-, meta-, and para-positions on the ring are here reported. The amino-nitrogen substitutions were methyl, ethyl, benzyl, and di-methyl. Of the 14 amines studied, 'Orthoxine' was chosen as the most interesting, since it had a low toxicity, high bronchodilator activity, practical lack of pressor action and was relatively easy to prepare synthetically. Consequently, a more detailed study was given this substance.

**CARDIOVASCULAR RESPONSES.** 1. *Blood pressure activity.* In many experiments with anesthetized dogs prepared for recording of blood pressure, the absence of significant pressor activity for 'Orthoxine' (EHW-286) was observed.

Typical responses are given in figures 1A and 1B. As can be seen in figure 1A, a 5 mgm. dose of 'Orthoxine' given intravenously to an atropinized dog, caused

TABLE 1

COMPOUND NUMBER	FORMULA	ACUTE TOXICITY L.D. <sub>50</sub> I.V. IN RATS mgm./kgm.	PRESSOR RATIOS IN DOGS. EPINEPHRINE ONE	BRONCHIAL RATING AGAINST PILOCARPINE CONSTRICCTION
1		60	1/1000	-2
2		40	1/250	6
3		30	1/250	4
4* Orthoxine		60	Depressor to 1/2000	12
5		35	1/500	5
6		40	1/225	2

\* Orthoxine.

TABLE 1—Continued

COMPOUND NUMBER	FORMULA	ACUTE TOXICITY L.D.50 I.V. IN RATS mgm./kgm.	PRESSOR RATIOS IN DOGS. EPINEPHRINE ONE	BRONCHIAL RATING AGAINST PILOCARPINE CONSTRICTION
7		40	Depressor	9
8		40	<1/200	10
9		50	1/1600	12
10		20	Depressor	9
11		20	1/2000	7
12		15	<1/2000	8

TABLE 1—Continued

COMPOUND NUMBER	FORMULA	ACUTE TOXICITY L.D.50 I.V. IN RATS	PRESSOR RATIOS IN DOGS EPINEPHRINE ONE	BRONCHIAL RATING AGAINST PILOCARPINE CONSTRICCTION
13		mgm./kgm. Material inadequate	>1/500	4
14		40	<1/350	1

a transient fall in arterial pressure of 18 mm. Hg. In this particular experiment the effect of 'Orthoxine' on histamine vasodepression also was recorded. The 5 mgm. dose caused a 36 per cent inhibition of the vasodepression produced by 2.5 micrograms of histamine. In figure 1B there is recorded a typical transient depressor response to a larger dose (25 mgm.) of 'Orthoxine'. This dose produced a fall in arterial pressure of 24 mm. Hg. The very definite inhibition of the pilocarpine response is noteworthy, but this property has not been investigated further. There was no appreciable effect of 'Orthoxine' on the pressor response to epinephrine.

The well-known pressor effect of ephedrine, observed by Chen and Schmidt (9), has been observed repeatedly in these as well as other laboratories. In both atropinized and non-atropinized dogs, ephedrine has 1/250 the pressor activity of epinephrine. 'Orthoxine', on the other hand, always produces slight vasodepression in atropinized dogs. In non-atropinized dogs either no effect on the blood pressure or slight increases were observed. In no experiment where atropine was not administered did the pressor response to 'Orthoxine' exceed 1/2000 that of epinephrine. 'Orthoxine' at a dose of 0.5 cc., 1:100, (5 mgm.) produced less pressor response than that produced by 0.25 cc., 1:100,000, (2.5 micrograms) of epinephrine. In most instances no pressor response was apparent. A vasodilator capacity of 'Orthoxine' is therefore evident which is masked by the intact parasympathetics. As soon as the latter are blocked by atropine only vasodepression is obtained, whereas ephedrine and other similar amines such as amphetamine ( $\beta$ -phenyl-isopropylamine) and 'Propadrine' ( $\beta$ -phenyl-isopropanolamine) always effect a pressor response.

2. *Heart rate studies.* Meek and Seevers (10) observed in a large group of dogs that intravenous doses of 1 to 5 mgm. of ephedrine per kgm. "almost always

resulted in initial bradycardia, shortly followed by peculiar grouping of beats with two or three cycles being separated by pauses." Heart rate and respiratory rate studies in these laboratories, comparing 'Orthoxine' with ephedrine, have substantiated the results of Meek and Seavers with ephedrine and, in addition, we have observed the effects of 'Orthoxine' on the normal dog. The results of one of these observations appear in table 2. It is evident from this table that at twice the dose of ephedrine, 'Orthoxine', in a dose of 10 mgm. per



FIG. 1. A. Dog blood pressure. Anesthetic = sodium phenobarbital I.P. A = Atropine sulfate, 1 mgm. per kgm. (10.5 mgm.). S = Epinephrine, 2.5 micrograms. H = Histamine base, 2.5 micrograms. EHW-286 = 'Orthoxine,' 5 mgm. Time interval = 5 seconds. B. Dog blood pressure. Anesthetic = sodium phenobarbital I.P. S = Epinephrine, 10 micrograms. Pilo = Pilocarpine hydrochloride, 1 mgm. EHW-286 = 'Orthoxine,' 25 mgm. Time interval = 5 seconds.

kgm. subcutaneously, did not appreciably alter the dog's heart rate. No bradycardia or arrhythmia was observed. Respiratory rate, however, was increased and the depth decreased. At 20 mgm. per kgm., or 4 times the ephedrine dose, 'Orthoxine' did produce the typical bradycardia that is observed with ephedrine at the smaller dose but no arrhythmia was noted. In fact the dog's heart rate definitely became more regular and appeared more forceful. Respirations were again fast and shallow.

**BRONCHODILATOR ACTIVITY.** Using the isolated rabbit lung method of Sollmann and von Oettingen, 'Orthoxine' was compared directly to ephedrine for its broncho-dilator properties.

1. *Pilocarpine bronchoconstriction.* When tested alone against pilocarpine constriction, 'Orthoxine' exerted a consistent and regular bronchodilator action represented by an average increase of 13 bubbles per minute.

TABLE 2  
*Heart rate and respiration studies*

EPHEDRINE SO <sub>4</sub> -5 MG.M./KGM.			ORTHOXINE--10 MG.M./KGM.			ORTHOXINE--20 MG.M./KGM.		
Time	Heart rate	Respirations per minute	Time	Heart rate	Respirations per minute	Time	Heart Rate	Respirations per minute
	beats per minute			beats per minute			beats per minute	
10:10	64*	20	1:45	61*	16	2:00	80*	20
10:12	68*	20	1:48	60*		2:02	78*	20
10:14	68*	20	1:51	60*	16	2:05	78*	20
10:16	68*	20	1:54	59*		2:08	72*	22
10:20	68*	20	1:57	60*	16	2:10	78*	22
Ephed. inj. 5 mgm./kgm. (51 mgm.)			2:00	60*		2:12	76*	22
			2:03	60*	16	2:15	76*	23
10:27	68†		Orthoxine inj. 10 mgm./kgm. (103 mgm.)			Orthoxine kgm. (206 mgm.)	inj. 20 mgm./kgm.	
10:29	68†	21	2:05	60	16	2:22	76	23
10:34		16	2:11		16	2:24	74	23
10:36	68†	21	2:15	56§	16	2:27	72	29
10:38	68†	21	2:18	58§	16	2:30	72	
10:40	60†	22	2:21	53§	16	2:32	68	30‡
10:42	50†	20	2:25	56§	16	2:34	68	30‡
10:43	48†		2:27	56§		2:36	62§	32‡
10:44	48†	21‡	2:28	58§		2:37	62§	34‡
10:47	46†	22‡	2:30	58§	22‡	2:40	62§	
10:50	40†	18‡	2:33	56§	22‡	2:45	60§	36‡
10:51		17‡	2:35		22†	2:51	60§	42‡
10:53	40†	18‡	2:38	56§		2:58	56§	50‡
10:55		18‡	2:44	56§	26‡	3:01	56‡	
10:57		20†	2:46	58§	24‡	3:10	58§	42‡
10:59	41†	24‡	2:48	56§		3:15	58§	37‡
11:03	40†	32‡	2:50	60§	28‡	3:30	62§	32‡
11:06	40†	32‡	2:53	56§	30‡	3:45	62§	33‡
11:10	41†	30‡	2:55	58§	32‡	3:47	60§	32‡
11:15	40†	28‡	2:58	56§	32‡	3:51	60§	
11:20	40†	32‡	3:00	56§	32‡	3:55	62§	32‡
11:23	40†	34‡						
11:25	40†	32‡						
11:30	40†	32‡						

\* Irregular.

† Irregular—in groups of 3 beats.

‡ Fast and shallow.

§ Regular and stronger.

Figure 2 shows the direct comparison of 'Orthoxine' and ephedrine against pilocarpine bronchoconstriction. The average increase in bubbles per minute produced by 'Orthoxine' equals 12, while comparable doses of ephedrine pro-

duced a bronchodilation represented by an average increase of 5.5 bubbles per minute. By this method, therefore, 'Orthoxine' is about twice as effective as ephedrine against pilocarpine bronchoconstriction and 1/20 as effective as epinephrine.

2. *Histamine bronchoconstriction.* Against histamine bronchoconstriction 'Orthoxine' was consistently superior to ephedrine as a bronchodilator. Figure 3 depicts the results of one of these experiments. In this chart the bronchodilation produced by 5 and 10 mgm. doses of 'Orthoxine' is represented by an average increase in bubbles per minute of 7, while ephedrine at comparable doses produced an average increase of only 1. From several comparisons against his-

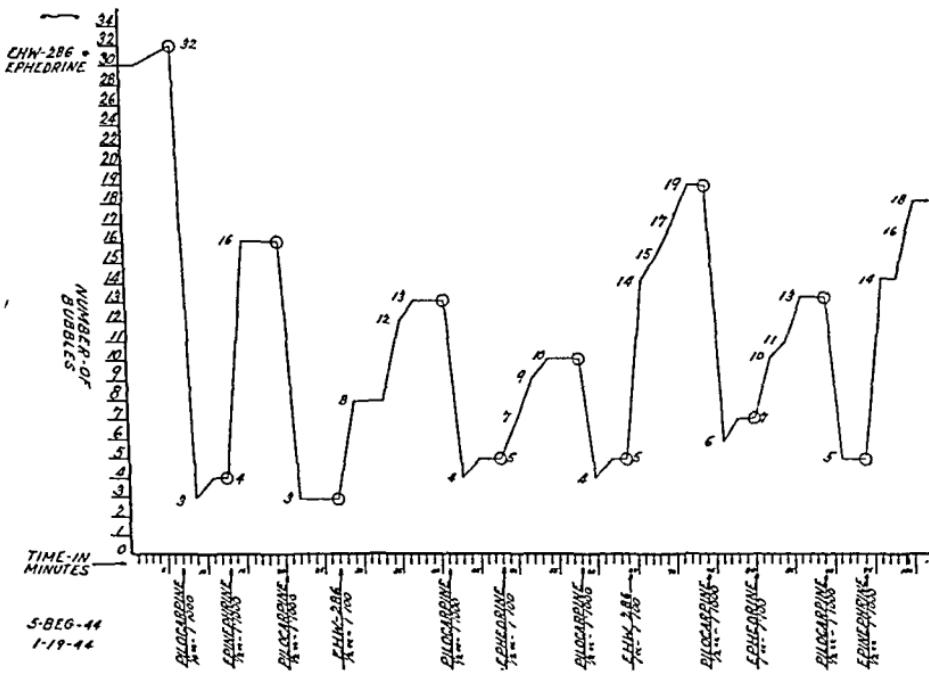


FIG. 2

tamine, 'Orthoxine' was found to be more than twice as effective as ephedrine and about 1/5 as effective as epinephrine.

3. *Acetylcholine chloride bronchoconstriction.* The bronchodilator effect after acetylcholine also was determined in several experiments. If responses to all doses are averaged, 'Orthoxine' is of about the same activity as ephedrine. If one compares only initial responses obtained at the time of greater sensitivity, 'Orthoxine' consistently was found more active than ephedrine against acetylcholine bronchoconstriction. Furthermore, against acetylcholine as against histamine, 'Orthoxine' is about 1/5 as active as epinephrine.

ISOLATED SMOOTH-MUSCLE TESTS. 1. *Histamine spasms.* Employing the isolated smooth-muscle test (method of Magnus), using strips of guinea pig

jejunum, 'Orthoxine' was compared directly to ephedrine for its effectiveness in blocking the spasmogenic activity of histamine. The results of this study indicate that 'Orthoxine' is approximately 8 times more effective than ephedrine as a histamine antagonist (figure 4). However, this activity should not be confused with that of the more powerful antihistaminics, since 'Orthoxine' was found to be about one-twentieth as effective as 'Benadryl' against histamine spasm of the guinea pig jejunum.

2. *Acetylcholine spasms.* It has been substantiated that sympathomimetic substances are not only effective histamine antagonists, but also antagonize acetylcholine spasms. Experiments similar to those above were carried out on

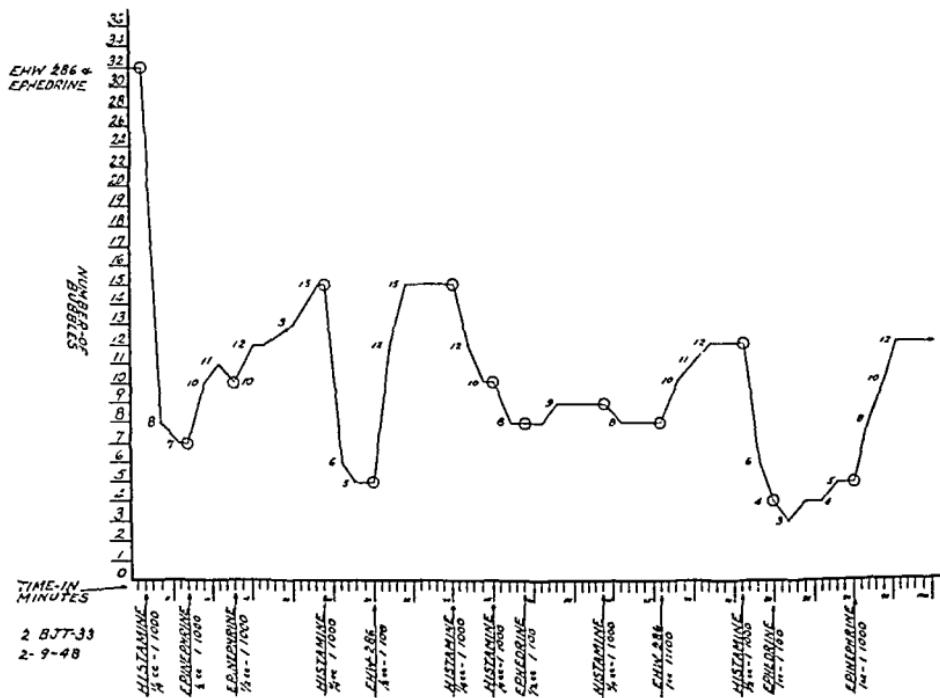


FIG. 3

the isolated rabbit's ileum employing acetylcholine as the spasmogenic agent. Figure 5 shows the results of one of these experiments. It is evident from this figure that 'Orthoxine' is 4 times as active as ephedrine in relieving acetylcholine-induced spasms. This definite degree of activity against acetylcholine spasms is of interest, but it should be emphasized that 'Orthoxine' is not a general anticholinergic drug since it is only 1/1000 as active as atropine in blocking acetylcholine responses.

3. *Barium chloride spasms.* The antagonistic action of 'Orthoxine' and ephedrine against barium chloride spasm of the rabbit's ileum is shown in figure 6. These results indicate that 'Orthoxine' is about 6 times more potent than ephed-

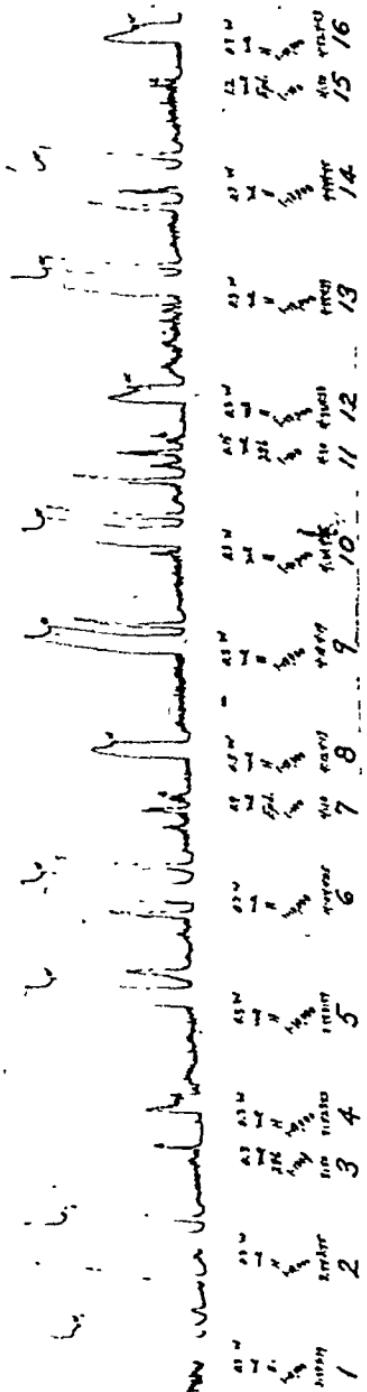


FIG. 4. GUINEA PIG JEJUNUM  
Injections #1, 2, 4, 5, 6, 8, 9, 10, 12, 13, 14, 16 = histamine base, 30 micrograms. Injection #3 = 'Orthoxine,' 3.0 mgm. Injection #7 = edrine, 9.0 mgm. Injection #11 = 'Orthoxine,' 1.5 mgm. Injection #15 = ephedrine, 12.0 mgm.

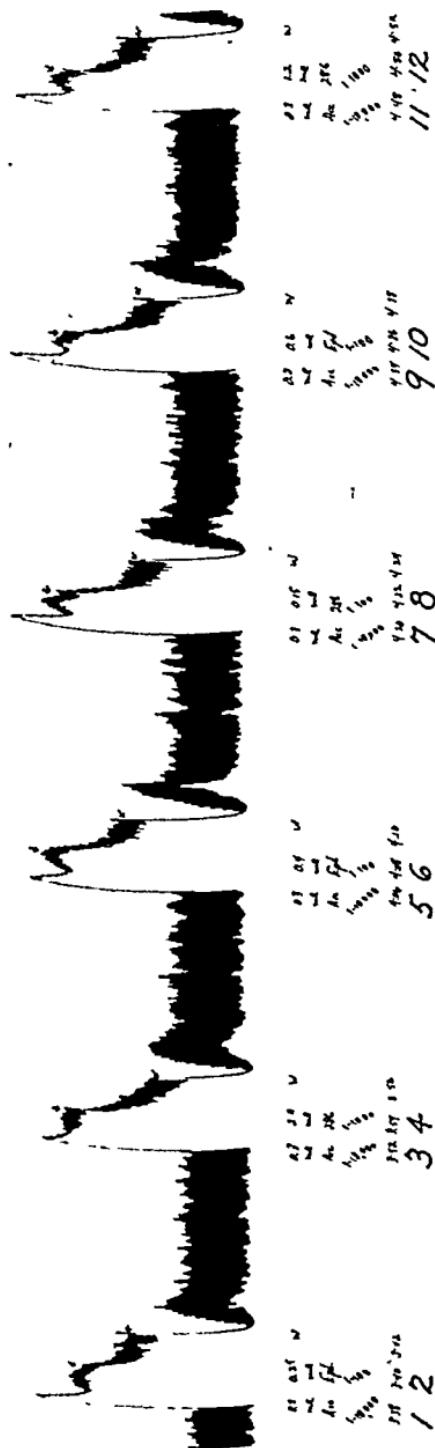


FIG. 5. RABBIT ILEUM  
Injections #1,3,5,7,9,11 = acetylcholine, 30 micrograms. Injection #2 = ephedrine, 2.5 mgm. Injection #4 = 'Orthoxine' 2.0 mgm.  
Injection #6 = ephedrine, 4.0 mgm. Injection #8 = 'Orthoxine', 1.5 mgm. Injection #10 = ephedrine, 6.0 mgm. Injection #12 =  
'Orthoxine', 1.2 mgm.

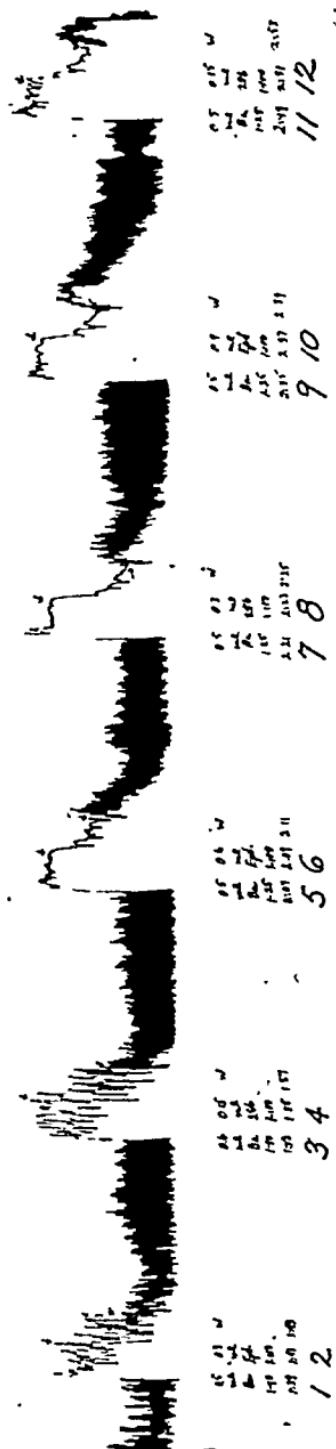


FIG. 6. RABBIT ILEUM  
 Injections #1, 3, = barium chloride, 10.0 mgm. and 12.0 mgm. Injections #5, 7, 9, 11 = barium chloride, 20.0 mgm. Injection #2 = ephedrine, 3.0 mgm. Injection #4 = 'Orthoxine,' 1.5 mgm. Injection #6 = ephedrine, 6.0 mgm. Injection #8 = 'Orthoxine,' 3.0 mgm. Injection #10 = ephedrine, 9.0 mgm. Injection #12 = 'Orthoxine,' 1.5 mgm.

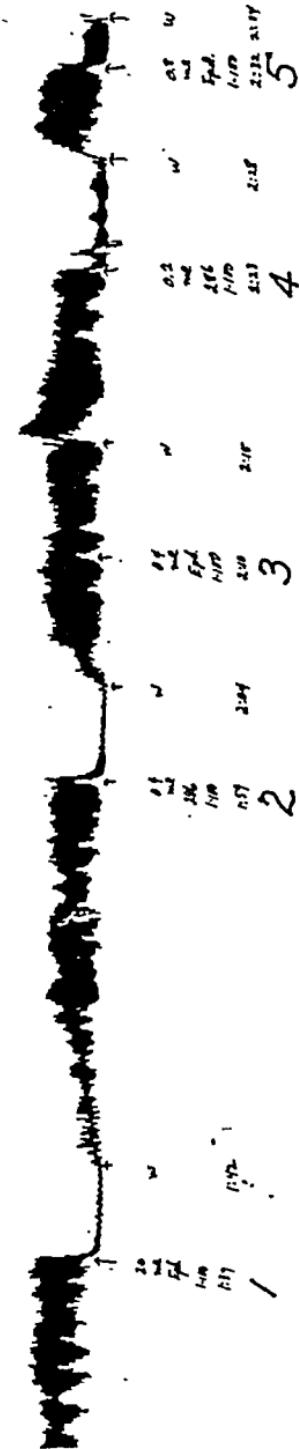


FIG. 7. UNSTIMULATED RABBIT ILEUM  
Injection #1 = ephedrine, 20.0 mgm. Injection #2 = 'Orthoxine,' 4.0 mgm. Injection #3 = ephedrine, 4.0 mgm. Injection #4 = 'Orthoxine,' 2.0 mgm. Injection #5 = ephedrine, 8.0 mgm.

rine. Compared to epinephrine, 'Orthoxine' is about one-tenth as effective a barium chloride antagonist.

In causing relaxation of the normal contractions of the unstimulated ileum of the rabbit, 'Orthoxine' was found to possess about 5 times the activity of ephedrine.

On the non-pregnant rabbit uterus, 'Orthoxine' was found to be only one-half as effective as ephedrine in stimulating the uterus to contraction.

*Deamination studies.* Employing the *in vitro* method of Beyer and Govier (11), enzymic oxidative deamination studies using guinea pig-liver homogenate were carried out in these laboratories.<sup>1</sup> 'Orthoxine', ephedrine, and several *n*-propylamines were tested. The results indicate that 'Orthoxine', like ephedrine, is not deaminated, while the straight-chain amines are deaminated oxidatively. It is reasonable to assume, therefore, in view of the literature reports and studies in these laboratories, that 'Orthoxine', being an isopropylamine, would exhibit activity when administered orally.

TABLE 3  
*Summary of acute toxicity data*

SPECIES	ROUTE OF ADMINISTRATION	LD 50 (MG/M <sub>2</sub> KGM)	
		Orthoxine	Ephedrine
Rats	Intravenous	50	102
Rabbits	Intravenous	>30-<35	73
Rats	Oral	630	404
Rabbits	Oral	652	825
Rats	Subcutaneous	573	318
Rabbits	Subcutaneous	269	383

*Acute toxicity.* The comparative evaluation of the toxicity of 'Orthoxine' and ephedrine was made in rats and rabbits by intravenous, subcutaneous, and oral administration. All rats used were adult males with an average body weight of 200 to 300 grams. All rabbits used were adults of both sexes with an average body weight of 2 kgm. Toxic signs exhibited by both species at high doses of both drugs consisted of violent convulsive seizures, hyperpnea, with partial or complete motor paralysis, followed by prostration. Animals that recovered from the more toxic doses usually did so in 6 to 8 hours following administration of the test substance. Both 'Orthoxine' and ephedrine, at the higher doses given subcutaneously to rats and rabbits, caused severe cutaneous and subcutaneous tissue damage at the injection site.

The results of this study are found in table 3.

*Chronic toxicity.* Chronic toxicity studies were carried out using rats, rabbits, and guinea pigs. 'Orthoxine' and ephedrine were administered to parallel groups of animals daily for 21 consecutive days. The rats (200- to 300-gram males) and rabbits (2 kgm.; both sexes) received the drugs orally and subcutaneously.

<sup>1</sup> The authors are indebted to Dr. W. M. Govier for these determinations.

The guinea pigs were administered the drugs subcutaneously only. The daily oral doses of both 'Orthoxine' and ephedrine administered to the rats and rabbits were 100 mgm. per kgm. of body weight. The daily subcutaneous doses in all three species were 20 mgm. per kgm. of body weight. The details of these chronic toxicity studies appear in table 4.

Immediately following the 21-day test period all the animals of all groups were subjected to autopsy, were examined grossly and were compared to the controls.

TABLE 4  
*Chronic toxicity table—21 days*

COMPOUND	ROUTE OF ADMINISTRATION	ANIMAL SPECIES	NO. OF ANIMALS	DOSE/KGM.	AVG. GAIN/ANIMAL IN 21 DAYS	RESULTS	REMARKS
Orthoxine	oral	rats	10	100	loss—35	all survived	
Orthoxine	oral	rats	10	100	6.8	all survived	
Ephedrine SO <sub>4</sub>	oral	rats	10	100	loss—22	1/2 survived	
Controls	—	rats	10	—	18.7	all survived	
Controls	—	rats	10	—	56	all survived	
Orthoxine	oral	rabbits	4	100	363	1/4 survived	
Orthoxine	oral	rabbits	4	100	370	all survived	
Ephedrine SO <sub>4</sub> ..	oral	rabbits	4	100	390	1/4 survived	
Controls	—	rabbits	4	—	375	all survived	
Controls	—	rabbits	4	—	351	all survived	
Orthoxine	sub cu	rats	10	20	56.2	all survived	severe tissue damage at inj. site
Ephedrine SO <sub>4</sub> .	sub cu	rats	10	20	53.5	all survived	" "
Controls	—	rats	10	—	56	all survived	
Orthoxine	sub cu	rabbits	3	20	366	all survived	severe tissue damage at inj. site
Ephedrine SO <sub>4</sub>	sub cu	rabbits	3	20	270	all survived	" "
Controls	—	rabbits	4	—	351	all survived	
Orthoxine	sub cu	guinea pigs	5	20	88	all survived	severe tissue damage at inj. site
Ephedrine SO <sub>4</sub> .	sub cu	guinea pigs	5	20	36	all survived	" "
Controls	—	guinea pigs	5	—	92	all survived	

Tissue samples of heart, lung, liver, spleen, stomach, duodenum, kidney, adrenals, pancreas, subcutaneous and skeletal muscle tissue were preserved for microscopic pathologic studies. Gross examination revealed nothing abnormal except for the severe cutaneous and subcutaneous tissue damage at the injection sites of all animals administered the drug by the parenteral route. Complete histopathologic examinations were made by Dr. A. James French, associate professor of Pathology, University of Michigan Medical School at Ann Arbor, Michigan. No pathological changes were apparent in the tissues of rabbits,

rats, or guinea pigs which had received either the 20 or the 100 mgm. per kgm. doses of 'Orthoxine', since there were no differences from like-tissues of control animals or those that had received ephedrine.

#### SUMMARY

In the study of a large series of phenyl propylamines several members of the group were found to possess greater bronchodilator activity than ephedrine with little or no pressor action. According to stability and activity the methoxy-phenylisopropylamines seemed most interesting. The intravenous toxicities, pressor actions, and bronchodilator properties of 14 of these amines are reported. Ortho-methoxy- $\beta$ -phenylisopropyl methylamine hydrochloride ('Orthoxine'), because of its relatively high activity and low toxicity, was investigated further with the following results:

1. In perfusion experiments with isolated lungs using pilocarpine, histamine, or acetylcholine as the constrictor agents, 'Orthoxine' was found to be on the average a much more effective agent than ephedrine for relieving bronchoconstriction. Against pilocarpine and histamine it is more than twice as effective as ephedrine and about 1/5 to 1/20 as effective as epinephrine.
2. When tested for activity in relieving intestinal smooth-muscle spasms, using isolated strips of jejunum or ileum, 'Orthoxine' was found to be 4 to 8 times as effective as ephedrine against the spasmogenic agents, histamine, acetylcholine and barium chloride. Compared to epinephrine, 'Orthoxine' is about 1/10 as effective a barium chloride antagonist. In quieting the normal contractions of the unstimulated intestinal muscle, 'Orthoxine' is 5 times as effective as ephedrine. On the non-pregnant uterus, 'Orthoxine' is only 1/2 as active as ephedrine in stimulating the muscle to contraction.
3. 'Orthoxine' produces little or no pressor response, possessing at most in this regard 1/2000 the activity of epinephrine and 1/8 that of ephedrine. Four times as much 'Orthoxine' as ephedrine must be administered to normal dogs to produce the bradycardia characteristic of the latter.
4. Inhibition of histamine bronchoconstriction, vasodepression, and smooth-muscle spasm shows that 'Orthoxine' possesses antihistaminic properties. The intestinal smooth-muscle tests indicate that the order of activity is 1/20 that of 'Benadryl' but is much greater than that of ephedrine.
5. Except for a higher intravenous toxicity, acute and chronic toxicity tests in rats, rabbits and guinea pigs show that the toxicity of 'Orthoxine' is of the same order as that of ephedrine. No pathological changes were found after the continuous daily administration of either 20 or 100 mgm. per kgm. doses for 21 days.
6. The pharmacological properties of 'Orthoxine' suggest that it may be of value in the treatment of asthma, for which it is presently being clinically evaluated.

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# THE ABSORPTION AND ELIMINATION OF CARONAMIDE (4'-CARBOXYPHENYLMETHANESULFONANILIDE)<sup>1</sup>

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An hypothesis that it should be possible to inhibit the renal tubular excretion of penicillin by an orally active compound that would be essentially refractory to elimination by that mechanism has been presented (1). Caronamide<sup>1</sup> (4'-carboxyphenylmethanesulfonanilide) was selected as being a compound that fitted these specifications closely. Recent clinical studies have confirmed its usefulness for the enhancement of penicillin plasma concentrations and therapy (2, 3). The uniformity of results in these reports and the apparent lack of renal toxicity of the drug have been gratifying.

Although the effects of caronamide on the excretion of the various penicillins and other agents have been published (4, 5, 6), a precise study of the absorption, distribution, and elimination of the compound has had to await definitive analytical methods. At the present the methods of Brodie, Levy, and Bernstein (7) and of Ziegler and Sprague (8) are the most satisfactory. They can be used to recover quantitatively caronamide added to body fluids, particularly plasma and urine (5). However, their lack of specificity in the differentiation of the drug from its real and likely metabolic products lends an element of uncertainty to the present interpretation of results.

This report deals with 1) the absorption and excretion of caronamide and its metabolite(s) following its oral administration; 2) the falling plasma concentrations of these compounds and their urinary recovery following intravenously administered caronamide, alone and as compared to mannitol; and 3) the distribution and falling plasma concentration of caronamide, its metabolite(s), and mannitol following intravenous administration to two-stage bilaterally nephrectomized dogs.

**METHODS.** A consideration of analytical methods used in this research is indicated because of their recent origin and the fact that in the course of metabolism studies they do not necessarily measure the same entity.

The method of Ziegler and Sprague (8) depends on the hydrogenolytic (catalytic) cleavage of the sulfonanilide linkage in caronamide to yield p-aminobenzoic acid which in turn is diazotized and coupled with N(1-naphthyl)-ethylenediamine dihydrochloride. The resulting colored compound is read colorimetrically at a wave length (or filter) of 540 millimicrons. In this report it will be referred to by the authors' names or as the colorimetric method. Following the administration of caronamide to animals the drug concentration determinations in body fluids are invariably higher by this method than the corresponding

<sup>1</sup> 'Staticin' is the trademark applied to 4'-carboxyphenylmethane-sulfonanilide, caronamide, by Sharp and Dohme, Inc.

values obtained by the method of Brodie et al. (7). If caronamide is precipitated from urine at pH 3 there remains in the urine of the patient or dog receiving the drug a compound or compounds that will react to this colorimetric method. This means that there is at least one metabolite of caronamide in which the sulfonanilide linkage has not been split and that is more soluble than the drug *per se*. No positive test for a free p-amino group, as in p-aminobenzoic acid, is obtained in the urine under these circumstances. From the urinary recovery figures in the following data it will be apparent that this method determines caronamide and its metabolites, i.e. *total* caronamide, in body fluids.

The method of Brodie, Levy, and Bernstein (7) depends on the fact that caronamide can be extracted into chloroform from an acidic aqueous medium and in turn reextracted from chloroform into 0.1 N sodium hydroxide. The alkaline solution then is read spectrophotometrically in the ultraviolet range (280.5 m $\mu$ ). It will be referred to herein as the Brodie or spectrophotometric method. Although it gives lower caronamide values in metabolic studies than the colorimetric method there is no present assurance that it measures caronamide to the exclusion of all metabolites. For example, we have found that the glycine conjugate of caronamide (p-phenylmethanesulfonamidohippuric acid), which conceivably could be a metabolite of caronamide, can be determined in this manner. Furthermore the conjugate is more soluble but much less active than is caronamide.

With the full knowledge that neither method probably measures definitively any one compound we have employed the terms *total* caronamide and caronamide *per se* to apply loosely to the measurements made and reported herein by the Ziegler-Sprague and the Brodie methods, respectively. It becomes apparent that one might expect a considerable variation in the data obtained under these circumstances. This has been minimized by the strictest attention to detail and experience on the part of the analysts in using the methods. Our recovery data using these methods have been reported previously (5).

The mannitol method used in this research was that of Smith, Finkelstein, and Smith (9).

**EXPERIMENTAL.** *The oral administration of caronamide results in rapid and practically complete absorption of the compound, as evidenced by the plasma concentration and urinary recovery.*

Plasma concentration data for one or more experiments on six dogs that were given 60 mgm./kgm. of caronamide are summarized in figure 1. The average values and the standard deviations therefrom represent the points on the curve as determined for caronamide *per se* and *total* drug. The area between the two curves has been cross-hatched to indicate the considerable amount of caronamide metabolite.

This rapidity of absorption seems characteristic of the compound for various species including man. In therapy it is customary to give an oral dose of caronamide at the time of the intramuscular injection of penicillin. Actually Verwey and Miller (10) used the simultaneous oral caronamide-intramuscular penicillin dosage scheme to demonstrate the therapeutic advantage of the use of caronamide with penicillin in experimental pneumococcal and typhoid infections in mice.

It is apparent that a metabolite of caronamide is formed rapidly and appears in substantial amounts at the time the first blood level determinations are made. The percentage of metabolite tends to increase with the falling blood level value for caronamide. This would make it appear that the over-all elimination of caronamide (inactivation plus excretion) is greater than for the sum of its metabolites.

*The urinary recovery of caronamide following its oral administration was essen-*

tially complete within 24 hours, as represented in table 1. On the average, all of a given dose of caronamide was recovered in the urine within 24 hours. Approximately 43 per cent of the total amount excreted appeared in the urine within 4 hours and about 60–70 per cent of the total drug excreted at 4 or 24 hours was caronamide, *per se*. Since the ratio of caronamide to total caronamide was essentially the same at 4 and 24 hours (incomplete and complete recovery), one may conclude that the overall rates of excretion of the two species of compound were similar.

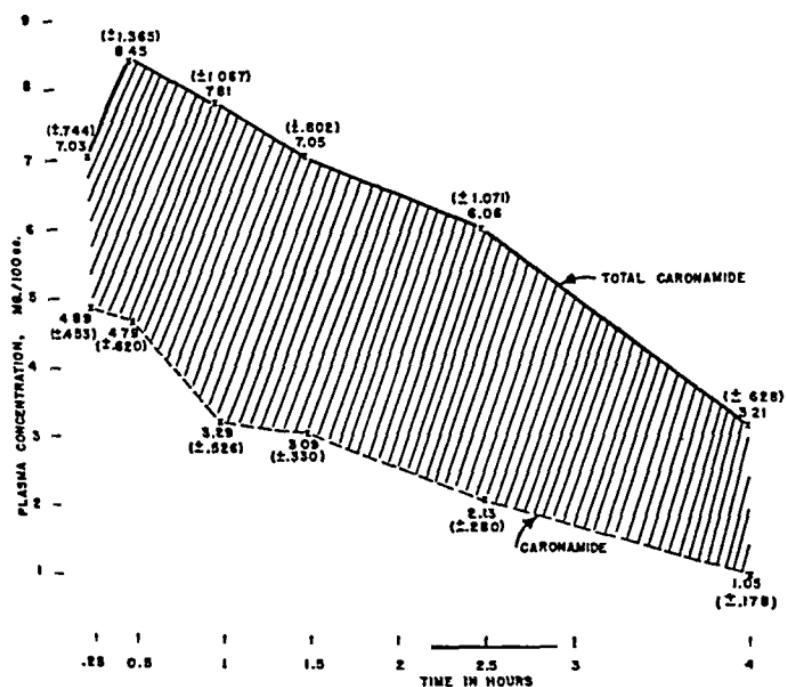


FIG. 1. AVERAGE PLASMA CONCENTRATION OF CARONAMIDE AND TOTAL CARONAMIDE FOLLOWING ORAL ADMINISTRATION OF SINGLE 60 MGMM./KGMM. DOSE TO EACH OF 6 DOGS

The coordinates represent the average value and standard deviation (s.d.) at each time interval.

As a check on the total recoveries, the urine was adjusted to pH 3 with acetic acid to precipitate caronamide (5). The precipitate was washed, analyzed for caronamide, and subjected to a melting point determination. The amount of caronamide metabolite remaining in the filtrate was determined colorimetrically. The sum of the values for precipitable and non-precipitable drug is represented in column 5 of table 1. Any differences between the data for total drug by the two methods probably may be accounted for on the basis of experimental error.

The falling plasma concentrations of caronamide and mannitol following intravenous administration were determined for five dogs and twice in two of the animals. The dosage of caronamide was 60 mgm./kgm. and that of mannitol

was 500 mgm./kgm. The drugs were administered intravenously as a single solution over a 5-minute period. This was tolerated well by the dogs. The data for the slopes of the mannitol, caronamide, and total caronamide, and the ratios of the caronamide:mannitol slopes have been summarized in table 2. Figure 2 presents the falling plasma concentration curves for the experiment that most closely represents the average of the data in table 2.

The average slope of the caronamide curves was essentially the same as for mannitol (av. slope ratio = 1.09). In the case of total caronamide the ratio

TABLE 1

*The percentage recovery of caronamide and its metabolite(s) following the oral administration of a single 60 mgm./kgm. dose of the drug.*

DOG	HOURS AFTER ADMINISTRATION	CARONAMIDE AND METABOLITES		
		Whole urine determinations		Precipitable drug* + non- precipitable drug†
		Caronamide	Total caronamide‡	
58	4	25.4	27.1	29.0
	24	69.7	118.5	106.0
	4	33.1	52.3	53.8
	24	55.6	86.5	84.6
	4	39.8	60.4	65.6
	24	64.0	(95.8)§	95.8
	4	27.7	34.6	36.6
	24	72.1	108.8	100.4
246	4	30.8	40.5	55.4
	24	46.0	91.7	92.6
average	4	32.6	42.9	48.1
	24	61.5	101.4	95.9

\* Precipitated at pH 3. From colorimetric assay and melting point determined to be essentially caronamide.

† Colorimetric assay of metabolites in the filtrate of urine following precipitation of caronamide ( $1 + 2 = 3$ ).

‡ The colorimetric determination of caronamide and its metabolite(s) in whole urine.

§ Not averaged.

to mannitol did not exceed 1.0, the average being 0.77. The greater average slope for caronamide than total caronamide is consistent with the conclusions drawn from the falling plasma concentration and urinary recovery data. Since the relative rates of excretion were the same and some metabolism of the compound obviously occurred, the slope of the caronamide curve must be the greater as a reflection of its combined metabolism and excretion.

The slope of the falling mannitol curve probably may be taken conservatively as a function of glomerular filtration rate, although there is not an unanimity

TABLE 2

A summary of the slopes\* and the ratio† of the slopes for mannitol and caronamide falling plasma concentrations following intravenous administration of the agents simultaneously.

The slopes are expressed as per cent/minute

DOG	MANNITOL SLOPE	CARONAMIDE		TOTAL CARONAMIDE	
		Slope	Ratio	Slope	Ratio
240	1.04	.98	.94	.62	.60
58	1.23	1.10	.89	1.05	.85
285	1.32	1.69	1.28	1.32	1.00
84	1.51	1.47	.97	.85	.56
365	1.43	2.28	1.59	1.21	.85
285	1.21	1.26	1.04	1.21	1.00
58	1.24	.97	.78	.65	.52
Average.....	1.28	1.39	1.09	.99	.77

$$* \text{Slope} = \frac{2.303 \log M_1 - \log M_2}{T_2 - T_1} \times 100.$$

$$T_2 - T_1$$

$$\dagger \text{Ratio} = \frac{\text{caronamide slope}}{\text{mannitol slope}}$$

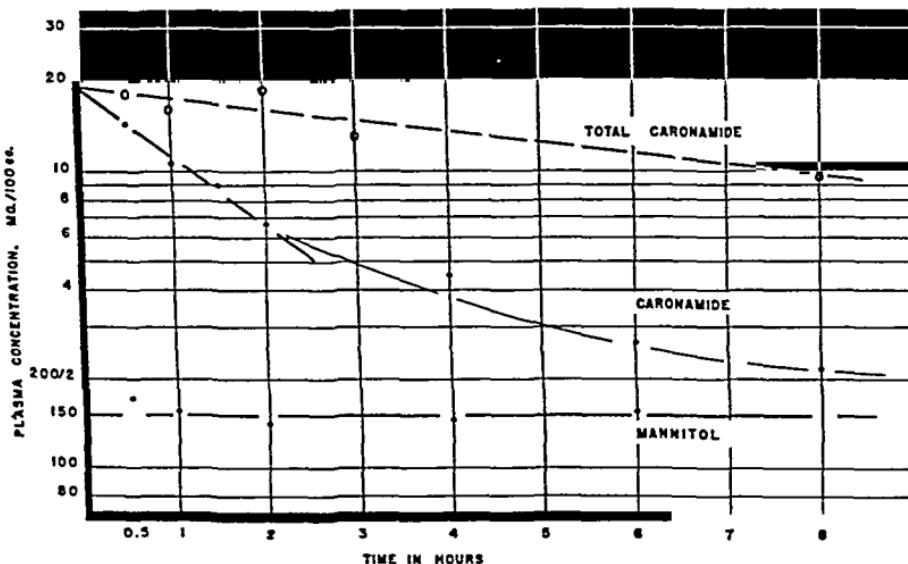


FIG. 2. FALLING PLASMA CONCENTRATIONS FOLLOWING SIMULTANEOUS INTRAVENOUS ADMINISTRATION OF CARONAMIDE (60 Mgm./Kgm.) AND MANNITOL (500 Mgm./Kgm.)

The data for the calculation of slopes and slope ratios in table 2 for dog 84 are presented in these curves.

of opinion as to the precision of this relationship (11). In confirmation of our previous findings (5), these data indicate that the overall elimination of caronamide (metabolism and excretion) is equivalent to the rate of elimination of

mannitol. The ratio of the slopes of two compounds that are eliminated solely by the kidney and of the same volume distribution should be equivalent to their clearance ratio. In this instance any influence of plasma binding on the elimination of the compounds is equated analytically into the coordinates from which the slopes are calculated. Therefore these data indicate that the rate of excretion of these compounds is no greater than the glomerular filtration rate.

The short-term urinary recoveries of intravenously administered caronamide and mannitol were determined as a check on the interpretation of their relative rates of excretion. The recoveries were performed over a 4-hour period on 5 dogs, 2 of them being used twice for this purpose. This time interval was selected as one wherein it was anticipated that the total excretion of each agent would not be completed, since this would invalidate any conclusion. A few values obtained

TABLE 3

*The percentage urinary recovery within 4 hours following the simultaneous intravenous administration of 60 mgm./kgm. caronamide and 500 mgm./kgm. mannitol.*

DOG NO.	MANNITOL	CARONAMIDE*	TOTAL CARONAMIDE†	TOTAL CARONAMIDE‡
58	74	39.0	80.0	71.2
58	112	49.0	66.0	—
240	99	31.2	60.5	63.0
84	88	53.0	92.6	87.7
285	81	45.0	70.1	76.7
285	52§	59.3	85.5	—
365	86	58.0	89.1	97.6
Average.....	90.0	47.8	77.7	79.2

\* Ultraviolet spectrophotometric method.

† Colorimetric method.

‡ Precipitable plus non-precipitable drug (see table 1).

§ Omitted from average.

over 2-hour collection periods did not alter the interpretation of the data presented in table 3 for the 4-hour recoveries.

The average total recovery of caronamide was of the same order (77.7 per cent) as for mannitol (90.0 per cent). The recovery of caronamide *per se* was about 60 per cent of the total recovery of drug (47.8 per cent caronamide). However individual comparisons of recoveries of the same compound in the same or different dogs and comparisons of the caronamide data with mannitol showed considerable variations. Thus the generalization made above from the average recovery figures is an approximation, subject to considerable variation.

The 24 hour urinary recovery of caronamide administered intravenously was determined following the injection of 60 mgm. of the drug per kgm. of body weight. The data for 6 dogs are presented in table 4. The analytical methods employed for the data in the three caronamide columns are the same as in the three previous tables.

Following intravenous administration the 4-hour urinary recoveries of caron-

amide and its metabolites (table 4) are greater than when the drug is administered orally but the ratio of the spectrophotometrically to the colorimetrically determined drug at 4 and 24 hours remains constant and is the same (about 60 per cent) regardless of the mode of administration. The total recovery of a given dose of caronamide as such and its metabolites is complete in 24 hours on the average, regardless of either oral or intravenous route of administration (tables 1 and 4).

TABLE 4

*The percentage urinary recovery of caronamide and total caronamide following the intravenous administration of a single 60 mgm./kgm. dose.*

DOG	HOURS AFTER ADMINISTRATION	CARONAMIDE*	TOTAL CARONAMIDE†	TOTAL CARONAMIDE‡
58	4	39.0	80.0	71.2
	24	55.4	117.0	120.0
240	4	31.2	60.5	63.0
	24	65.1	86.6	122.5
365	4	46.8	80.7	82.9
	24	50.3	104.0	110.2
365	4	58.0	89.1	97.6
	24	58.0	109.6	97.6
840	4	45.4	41.7	66.2
	24	45.4	74.4	86.2
84	4	53.0	92.6	87.7
	24	60.0	110.4	96.7
285	4	45.0	70.1	76.7
	24	54.0	95.8	85.7
Average	4	45.4	73.4	77.9
	24	55.4	99.7	102.7

\* Ultraviolet spectrophotometric method.

† Colorimetric method.

‡ Precipitable plus non-precipitable drug.

*The plasma concentration of caronamide and mannitol in bilaterally nephrectomized dogs was determined after the intravenous injection of the two compounds. The nephrectomies were performed in two stages on each of 4 well-trained dogs. From two weeks to several months following the first unilateral nephrectomy the second kidney was removed under intravenous thiobarbiturate induction and cyclopropane-oxygen closed-system maintenance anesthesia. The second operation was performed in the afternoon preceding the day of the test. The dogs were in good spirits and physical condition during the test.*

Free access to food and water was permitted following surgery and throughout the experiment. Since they were trained previous to surgery they submitted readily to venipuncture during the experiments.

The intravenous dosages of caronamide and mannitol were 60 and 500 mgm./kgm., respectively. Mannitol and caronamide plasma levels were followed in most instances for 8 hours. At the end of that time the dogs were sacrificed by anesthetization. The total elapsed time following nephrectomy was 24 hours. The data for a representative experiment are presented in figure 3.

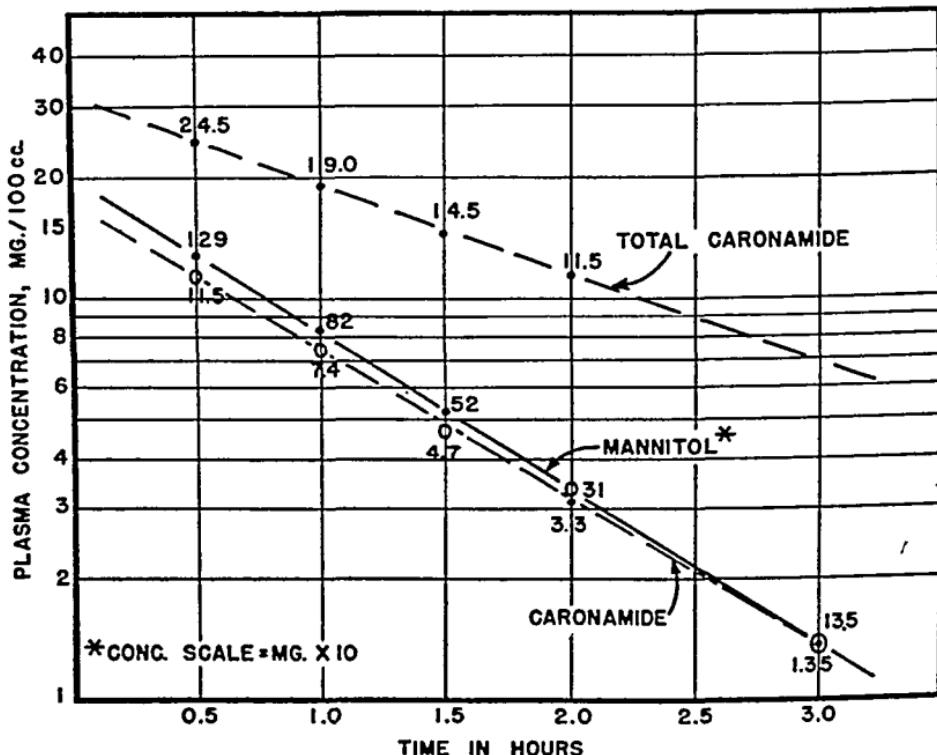


FIG. 3. PLASMA CONCENTRATION OF CARONAMIDE, TOTAL CARONAMIDE AND MANNITOL FOLLOWING INTRAVENOUS ADMINISTRATION OF 60 MG.M./KGM. OF CARONAMIDE AND 500 MG.M./KGM. OF MANNITOL TO BILATERALLY NEPHRECTOMIZED DOG WEIGHING 13.2 KGM.

After its distribution in the body the mannitol plasma concentrations fell but little or not at all over an 8-hour period in these 4 experiments, as illustrated in figure 3. The data would indicate that if any metabolism of mannitol occurs in the body—a controversial point (11, 12)—the site of action must be limited to the kidneys, since they are the only organs absent in this experiment.

Over the first 1½- to 2-hour period following the injection of caronamide the logarithm of its falling plasma concentration was a linear function of time. The slopes of the four caronamide curves (minus the slope of the total caronamide curve in two instances where this was greater than zero in the first two hours) were 0.540, 0.623 and 0.909; average 0.667. The semi-logarithmic char-

acteristic of the curve probably represents a first-order chemical reaction as describing the process of drug metabolism. In terms of average values, the slope of the curves representing the metabolism of caronamide in nephrectomized dogs is 48 per cent of the slope of the curve describing metabolism plus excretion of the drug in normal dogs.

During the first 2-hour period the value for *total* caronamide remains constant or falls but slightly. However, the total value for caronamide and its metabolites tends to decrease, in some instances considerably, after the first two hours. This is apparent in figure 3. The significance and even the validity of these values over a period of 4 to 8 hours is uncertain at present, since the values are erratic within an experiment and vary considerably among the animals. To a lesser extent this variation is true for the spectrophotometric determinations of caronamide.

The volume distribution of caronamide and mannitol have been calculated by extrapolating the plasma values to zero time and substituting in the equation  $V = M/C$  where  $V$  = volume distribution,  $C$  = plasma concentration, and  $M$  = total dose. In turn, the volume distribution was expressed as per cent of total body weight. This seems permissible for the mannitol since its slope is zero and for caronamide since its two curves intersect, as they should, at zero time. In each instance the volume distribution for caronamide was greater than for mannitol. In the four dogs the ratios of the percentage volume distribution of caronamide to mannitol were 1.10, 1.18, 1.38 and 1.44 (average 1.27). The significance of this difference is uncertain. In these experiments the range of the percentage volume distribution of mannitol among the dogs was from 22.6 to 29, which expressed similarly would give a ratio of 1.28.

The volume distribution of mannitol is reported to be equivalent to extracellular fluid volume (11a, 12b). From the above data, the volume distribution of caronamide may be said to be of the order of, or greater than extracellular fluid volume, although a more precise identification of the distribution of the drug with that fluid compartment would be beyond the scope or intent of these data.

**DISCUSSION.** A study of the absorption, metabolism, and excretion of caronamide has had to wait satisfactory analytical methods. To date the methods permit a separation of caronamide from another metabolite or other metabolites. It is evident throughout these experiments that metabolism of the compound takes place rapidly and to a considerable extent.

At present the identity of the metabolite(s) is not known. We do know that ordinarily the sulfonanilide linkage in caronamide is not broken by the body, for the overall urinary recovery of the drug and its metabolites having this linkage is essentially complete. However, in the metabolism of the compound something happens to increase its solubility so that the metabolite remains in solution at pH 3 or less where caronamide precipitates quantitatively. This increased solubility is important and is a fortunate circumstance. In the case of the sulfonamides, the metabolite (acetyl-sulfonamide) is usually less soluble than is the active compound. Insofar as they have been tested the possible metabolites of caronamide are considerably less active than the drug *per se*.

It is apparent that the absorption of the drug following oral administration is rapid and complete, from the data presented herein and from other experience dealing with patients. In collaboration with Dr. Wm. P. Boger we found no caronamide in the feces of patients to whom the drug was administered orally. However, we have had an opportunity to determine the plasma and urine concentration of caronamide in a child who did not respond with elevation of penicillin plasma concentration when the antibiotic agent was given intramuscularly and the adjuvant was administered orally. In this singular case the absorption of the drug was much reduced as judged by plasma concentrations and urinary recovery. This is the only instance of such a case that has come to our attention (13).

Some 43 per cent or so of a given dose of caronamide is excreted within four hours after its administration, normally. The total dose is excreted within a 12- to 24-hour period. The slope of the falling caronamide plasma concentration curve is the same as, and that for its metabolite is less than, that for mannitol. However, these slopes are a reflection of metabolism and excretion of caronamide, and the overall elimination of the drug and its metabolites; for the urinary output of the drug is considerably less than, and the excretion of total caronamide is about the same as that for mannitol. Other extensive studies of the renal clearances (14) and renal extraction (15) of caronamide and its metabolites will be reported in the near future.

The volume distribution of caronamide appears to be of the same order but somewhat greater than for mannitol in bilaterally nephrectomized dogs. In these animals the curve for the plasma concentration of caronamide falls in the manner of a first order chemical reaction over the first 1½ to 2 hours. Thereafter an equilibrium seems to be reached slowly between the drug and its metabolite(s), and there is some indication that the latter agent either is destroyed slowly or is more widely distributed in the body.

#### SUMMARY

Caronamide is rapidly and completely absorbed when administered orally to dogs, as judged by the plasma levels and the overall urinary recovery of the drug and its metabolite(s).

After oral or intravenous administration of caronamide to normal dogs or intravenous administration to bilaterally nephrectomized dogs a portion of the drug is metabolized rapidly. About 60 per cent of a dose of the drug administered to normal dogs is excreted, presumably as such. The other 40 per cent is excreted in the form of a metabolite(s) that is more water-soluble than is the parent compound. The distribution of caronamide in the body is of the same order as, but greater than, that for mannitol, whose volume distribution approximates extracellular water.

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# A COMPARISON OF ONE DOSE PER DAY WITH SIX DOSES PER DAY OF QUININE IN THE SUPPRESSION OF LOPHURAE MALARIA IN THE DUCK

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In previous experiments with lophurae malaria in the duck (1), it was shown that the minimal effective dose of quinine was smaller when the drug was administered once daily by stomach tube than when the drug was administered more or less continuously (drug-diet method). It might have been concluded, therefore, that a maintained plasma concentration was less effective than intermittent higher plasma concentrations. Two criticisms may be directed at these studies. First, drug administration was begun 18 hours before the birds were infected with *Plasmodium lophurae*, hence it was possible that the effect of treatment was attributable to the fact that the infection was not established before treatment was begun. Second, it was impossible with the drug-diet method to ascertain the dosage accurately. However, with the variety of drugs studied, all possible relationships between the single dose per day and the drug-diet method were found; therefore it appears improbable that the criticisms mentioned seriously affected the conclusions drawn. The present study was planned and carried out avoiding the two criticisms mentioned. Our previous conclusions were confirmed.

**METHODS.** White Pekin ducklings weighing from 110 to 560 grams were randomized into 10 groups. They were kept in standard poultry cages and were allowed free access to Purina Chick Starter Mash and running water. The ducklings were infected with *Plasmodium lophurae* by an intravenous injection of 0.25 cc. of blood containing  $150 \times 10^6$  parasitized erythrocytes. The course of the infection was followed by making a daily thin smear of the blood of each duck. The smears were counted as outlined previously (2, 3).

Treatment was given by stomach tube and was begun 24 hours following infection. Some groups of ducks received one administration of drug each day, while others received six administrations each day at intervals of four hours. All doses of quinine were calculated as base and were administered as an aqueous solution of the hydrochloride in a volume of 10 cc. per kgm. Treatment was continued for four days. Quinine was given in doses of 4, 8, 16 and 32 mgm. per kgm. once per day for four days and in doses of 2, 4, 8 and 16 mgm. per kgm. six times per day for four days. The ducks were weighed on the first and third days of treatment and individually dosed according to weight.

**RESULTS.** The data are shown in table 1. The minimal effective daily dose for the single dose per day is 8 mgm. per kgm. while for the multiple dose regimen it is 24 mgm. per kgm. In both of these groups the parasitemia of every duck was below .56 per cent erythrocytes parasitized whereas only 3 of the 17 controls evidenced a parasitemia less than .56 per cent erythrocytes parasitized. The probability that such a difference is a chance variation is extremely small. It

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appears, therefore, that the minimal effective dose of quinine was three times smaller for the single dose per day. It must be pointed out that 32 mgm. per kgm. in one dose per day was no more effective than 16 mgm. per kgm. and that the effect was comparable to 8 mgm. per kgm. given six times per day. It must be noted that six doses of 16 mgm. per kgm. per day produced a more marked reduction of parasitemia than did one dose of 32 mgm. per kgm. per day. The

TABLE 1

*A comparison of one dose per day with six doses per day of quinine in the suppression of lophurae malaria in the duck*

MGM./KG.M. PER DOSE	NUMBER OF DOSES	TOTAL DAILY DOSE	NO. OF DUCKS		PER CENT ERYTHROCYTES PARASITIZED				
					Days after infection				
					1	2	3	4	5
0	0	0	17	Mean* Range	2 0.6-3	3 1-5	12 4-22	43 20-62	56 8-69
2	6	12	9	Mean Range	1 0.4-3	3 1-6	15 4-22	40 18-55	50 24-67
4	6	24	9	Mean Range	2 0.8-4	2 1-8	7 1-19	20 1-42	30 0.7-54
8	6	48	9	Mean Range	1 0.07-4	0.8 0.01-3	0.7 0.01-3	0.5 0.01-2	0.3 0.01-2
16	6	96	9	Mean Range	1 0.7-2	1 0.8-4	0.5 0.3-1	0.3 0.09-0.6	0.04 0.02-0.2
4	1	4	9	Mean Range	1 0.6-2	3 1-5	10 6-18	30 19-42	50 38-55
8	1	8	9	Mean Range	1 0.6-2	1 0.7-7	2 0.6-18	3 0.2-32	3 0.3-43
16	1	16	9	Mean Range	1 0.6-3	1 0.6-2	0.8 0.3-2	0.2 0.04-0.6	0.2 0.04-0.7
32	1	32	9	Mean Range	0.9 0.5-2	1 0.6-2	0.6 0.4-1	0.2 0.1-4	0.2 0.1-5

\* The means given are geometric means.

meaning of this difference is not clear from these data; however, it may possibly be due to the duration of treatment.

There seems little doubt that the infection had become established during the 24-hour period before the beginning of treatment since the per cent of erythrocytes parasitized 24 hours after infection was higher than the calculated parasitemia at the time of infection in 83 of the 89 ducks used. It has been

shown previously that in this laboratory the calculated parasitemia coincides closely with the values obtained by counts made on blood smears taken immediately after infection.

DISCUSSION. These experiments confirm those published previously (1) but eliminate the criticism that treatment was begun before the infection was established. In addition, by administering the drug by stomach tube we have avoided the difficulty, inherent in the drug-diet method, of accurately determining the drug intake.

Repeated experiments using doses of quinine larger than those used in this study have shown that no fluorescent substance remains in whole blood (determined by the metaphosphoric acid precipitation method (4)) 24 hours after administration of the drug. This fact and the effectiveness of a single daily dose of quinine demonstrate that maintenance of a blood concentration is not necessary for effective suppression of lophuriae malaria in ducks. This evidence bears directly on the mechanism of action of the drug and is, therefore, of great theoretical interest. It is also of practical importance because of the greater simplicity and ease of the single dose a day regimen. It should be pointed out that testing of drugs for antimalarial activity using a single dose a day would be likely to miss drugs such as sulfonamides with which a maintained concentration is necessary. However, in screening by the drug-diet or multiple dose methods it is unlikely that one would miss drugs which are more effective in a single dose a day.

It has been shown in mice infected with  $\beta$ -hemolytic streptococcus, Group A, Strain C 203 and treated with penicillin that a single injection is as effective as an equal amount of drug divided into multiple injections given over a 24-hour period (5, 6). This is also true in mice infected with *Klebsiella pneumoniae* and treated with streptomycin (7) and in mice infected with Type I pneumococcus and treated with penicillin (8). That treatment can be omitted for periods as long as 12 hours without detriment to the human patient has been shown by Tillett in treating pneumococcal pneumonia with penicillin (9).

Our data, together with these studies, indicate that with quinine, penicillin and streptomycin, effective treatment does not depend on maintenance of the blood concentration. On the other hand, it has long been known that a maintained blood concentration is essential in chemotherapy with sulfonamides. It is obvious, then, that the optimal dosage regimen must be determined experimentally with each chemotherapeutic agent to insure the most effective use.

#### CONCLUSION

The daily dose of quinine effective in suppressing lophuriae malaria in the duck is approximately 3 times smaller when it is given in a single dose than when it is divided into 6 doses.

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# THE INFLUENCE OF LUNG DISTENSION UPON THE RESPONSE OF THE BRONCHIOLES TO EPINEPHRINE AND TO HISTAMINE

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During the course of experiments in which the changes in size of the bronchioles and the blood pressure were being recorded simultaneously, it was observed that following bronchoconstriction and fall in blood pressure induced by the injection of histamine, the bronchodilator response to epinephrine was seldom maximal, despite a good pressor response. In many such experiments also, with repeated administrations of epinephrine, the bronchioles developed a completely non-responsive or refractory state, so that it was no longer possible to obtain any further dilatation with epinephrine although again the blood pressure response was quite good. However, after the development of this refractory state, a good bronchodilator response to epinephrine could invariably be elicited by briefly distending the lungs by blowing directly into them through a tracheal cannula.

In view of this latter observation, which, as far as we know, has not been previously described, it was of interest to study these phenomena further. The object of this paper is to present the results of some of these studies with particular reference to the bronchiolar responses to epinephrine and to histamine, as affected by the existing degree of lung distension.

**METHODS.** Dogs, anesthetized with pentothal sodium<sup>2</sup> were used in all of these experiments. The animals were kept deeply anesthetized to the point of respiratory paralysis by repeated injections of the anesthetic agent.

The bronchiolar changes were recorded by the method described by Jackson (1), employing intermittent negative pressure ventilation after inserting the metallic chest plate. At the beginning of the experiment a tracheal cannula was inserted and artificial respiration temporarily maintained using a Starling pump. The vagus nerves were also cut. Blood pressure was recorded directly from the left common carotid artery.

The effects of histamine dihydrochloride and epinephrine hydrochloride were tested both by intravenous injection and by direct spraying into the lungs, using an ordinary DeVilbiss atomizer attached to an oxygen cylinder, and adjusted so that 1 cc. of the solution was aerosolized in 5 seconds. The duration of the spraying was never more than 10 seconds, and similar sprayings of physiological saline were without effect on the bronchioles. The outlet of the atomizer was inserted through a large tracheal cannula and reached just inside the trachea itself, so that all the spray was deposited within the tracheobronchial tree.

Brief measured continuous positive pressure was employed to obtain lung distension. This was accomplished by attaching the tracheal cannula to an air pressure system previously adjusted as desired. Briefly, compressed air entered the system through one arm of a Y-shaped arrangement, leaving through two outlets, one of which was connected to

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the tracheal cannula and the other to a piece of rubber tubing with a screw clamp. The pressure in the system was adjusted to the desired level by partially closing the outflow of air through this latter outlet. A water or mercury manometer was suitably attached to the tube going to the trachea.

At the beginning of an experiment the outlet for attachment to the tracheal cannula was closed with a hemostat, and the pressure in the manometer adjusted by turning on the compressed air moderately and partially closing the outlet with the screw clamp. A rate of air flow of 750 to 800 cc. of air in 5 to 10 seconds was generally sufficient to distend the dog's lung completely.

A pressure of 40 mm. of mercury was generally used. In other experiments however, positive pressures as low as 20 cms. of water were employed with much the same results provided the rate of flow of air into the lungs was stepped up and the bronchioles not too constricted. In addition, the distension of the lungs could be observed directly through the glass window in the Jackson's plate.

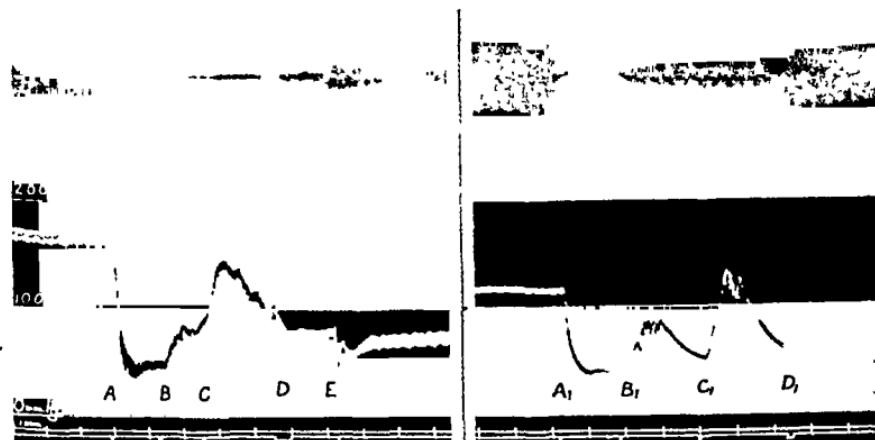


FIG. 1. Dog, male, 8.1 kgm. Pentothal sodium anesthesia. Vagi cut. Records of bronchioles (upper) and blood pressure (lower). A and  $A_1$ —histamine(0.62 mgm. per kgm.) intravenously. B and C—epinephrine (0.1 mgm.) and  $B_1$  and  $C_1$ , epinephrine (0.2 mgm.) intravenously. D—lung distension with positive pressure (20 cms.  $H_2O$  for 30 secs.).  $D_1$ —lung distension with positive pressure (40 mm. Hg for 10 secs.). E—lung distension by blowing directly through tracheal cannula. An interval of 20 minutes elapsed between E and  $A_1$ .

During the administration of positive pressure and of the spraying of the drugs into the lungs, it was necessary to clamp the rubber tubing connecting the tracheal cannula with the recording tambour, and this accounts for the gaps in the bronchiolar records corresponding to these procedures.

**RESULTS.** 1. *Effects of lung distension on the responses to injected epinephrine and histamine.* Figure 1 shows a typical example of the results obtained in several experiments. Thus, following intense bronchoconstriction and fall in blood pressure induced by the intravenous injection of histamine, two successive injections of epinephrine induced only slight bronchodilatation, although the blood pressure rose with each injection. Later, mild distension of the lungs using a positive pressure of 20 cms. of water for 30 seconds, produced some further bronchodilatation. However, complete distension of the lungs by forcibly

blowing directly through a piece of rubber tubing temporarily connected to the tracheal outlet, led to a much greater and sustained bronchodilatation. During the distension of the lungs the blood pressure fell but returned promptly almost to the preinjection level.

Later in the same experiment is shown a repetition of the histamine effects followed by two successive injections of epinephrine, each of which produced good pressor effects but only a moderate degree of bronchodilatation. It should be noted that the second epinephrine injection led to no further bronchodilatation, despite a good pressor response, that is to say, the bronchioles were now refractory to epinephrine. Two minutes later however, following lung distension there was a prompt and prolonged bronchodilatation.

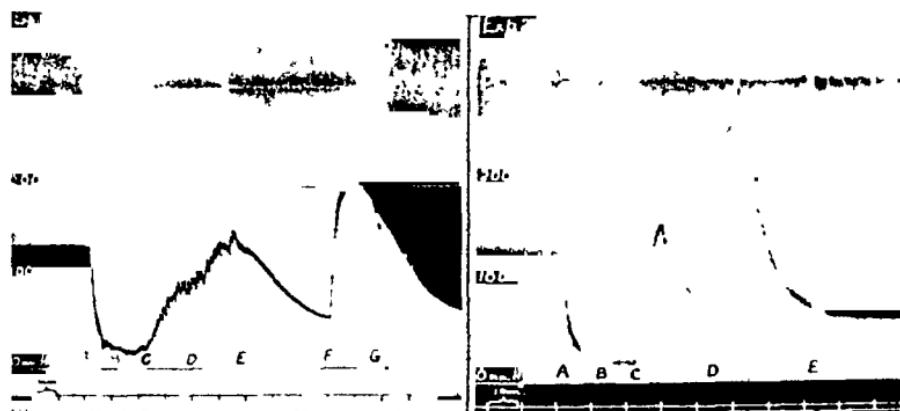


FIG. 2. Records of bronchioles (upper) and blood pressure (lower). *Exp. 1:* Dog, female, 5.4 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.7 mgm. per kgm.) intravenously. B—lung distension with positive pressure (40 mm. Hg for 10 secs.). C, D, and F—epinephrine (0.2 mgm.) intravenously. E and G—lung distension with positive pressure (40 mm. Hg for 5 secs.). *Exp. 2:* Dog, male, 7.05 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.7 mgm. per kgm.) intravenously. B and E—lung distension with positive pressure (40 mm. Hg for 10 secs.). C and D—epinephrine (0.2 mgm.) intravenously.

Similar results have now been obtained in 40 different experiments in which the lungs were either distended by blowing forcibly into them directly or by the use of various measured degrees of positive pressure and rate of air flow. All in all, the phenomenon appears to depend primarily on obtaining good lung distension. Thus, when low positive pressures such as 20 cms. of water were used, a higher rate of air flow was necessary.

It was of interest at the outset to determine whether or not similar lung distension alone could counteract the bronchoconstrictor action of histamine. Figure 2 shows results of two experiments in which, following intense bronchoconstriction and fall in blood pressure after intravenous injections of histamine, distension of the lungs produced only a slight and transitory bronchodilatation. Subsequent epinephrine injections in both experiments induced good blood pressure effects but only moderate bronchodilatation. Again, in both of these experiments there is evidence of refractoriness to the second epinephrine injection.

but following brief lung distension with the same degree of positive pressure as employed earlier in the experiments, there was a prompt and sustained bronchodilatation in each instance.

The dilatation of the bronchioles after epinephrine and lung distension appears to be maximal or almost maximal. Thus, as shown in exp. 1 (fig. 2) a further injection of epinephrine 3 minutes after the lung distension, although it induced a good pressor effect, led to no further bronchodilatation. On the contrary, two minutes later, distension of the lungs at G produced again as much dilatation as before. In many experiments this intense bronchodilatation continued for as long as 30 to 40 minutes, and the recovery was apparently complete.

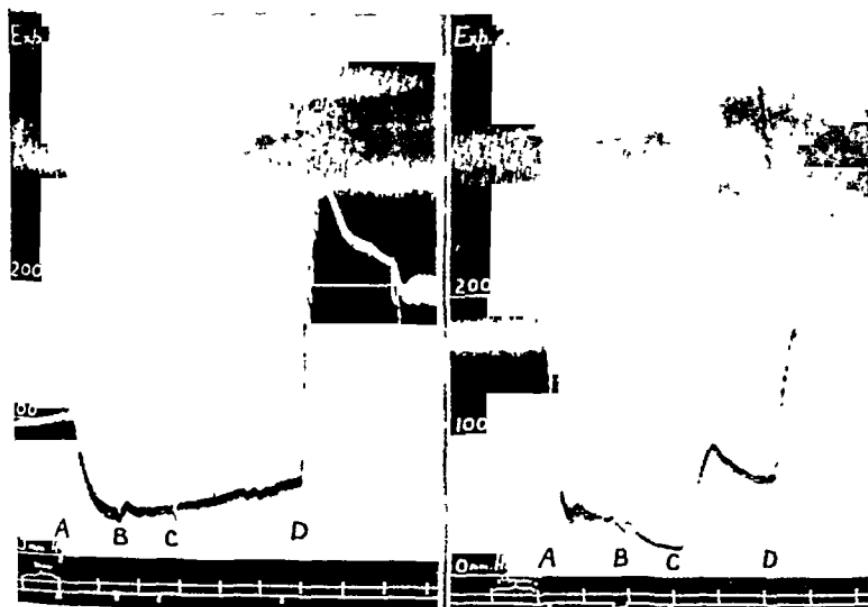


FIG. 3. Records of bronchioles (upper) and blood pressure (lower). *Exp. 1:* Dog, female, 12.2 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.35 mgm. per kgm.) intravenously. B—lung distension with positive pressure (20 mm. Hg for 5 secs.). C—lung distension with positive pressure (40 mm. Hg for 10 secs.). D—epinephrine (0.2 mgm.) intravenously. *Exp. 2:* Dog, male, 8.25 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.6 mgm. per kgm.) intravenously. B—lung distension with positive pressure (40 mm. Hg for 15 secs.). C and D—epinephrine (0.2 mgm.) intravenously.

In figure 3 are shown results of two other experiments in which the effects of lung distension in counteracting histamine bronchoconstriction were tested. Here again following injection of histamine (exp. 1) the first distension of the lungs caused little or no dilatation. However, a second more intense and prolonged distension of the lungs at C, caused a good bronchodilatation, lasting for several minutes. There was only a slight gradual increase in the blood pressure level. However, injection of epinephrine at D, induced the intense pressor response shown concomitant with marked and prolonged bronchodilatation. Somewhat similar results are shown in exp. 2 in the same figure. These need no further comment.

It appears from these and other experiments that when the bronchoconstrictor action of histamine is partially abolished by lung distension, the effects of injected epinephrine are greatly potentiated. This may account for the much better bronchodilator and pressor responses shown in figures 2 and 3, in contrast with those shown in figure 1, where the order was reversed. The mechanism of this effect is not clear.

It also appears from the above results that the degree of antagonism of histamine bronchoconstriction by lung distension depends on the relative intensity of the two actions. If this were so, it should be possible then, to antagonize

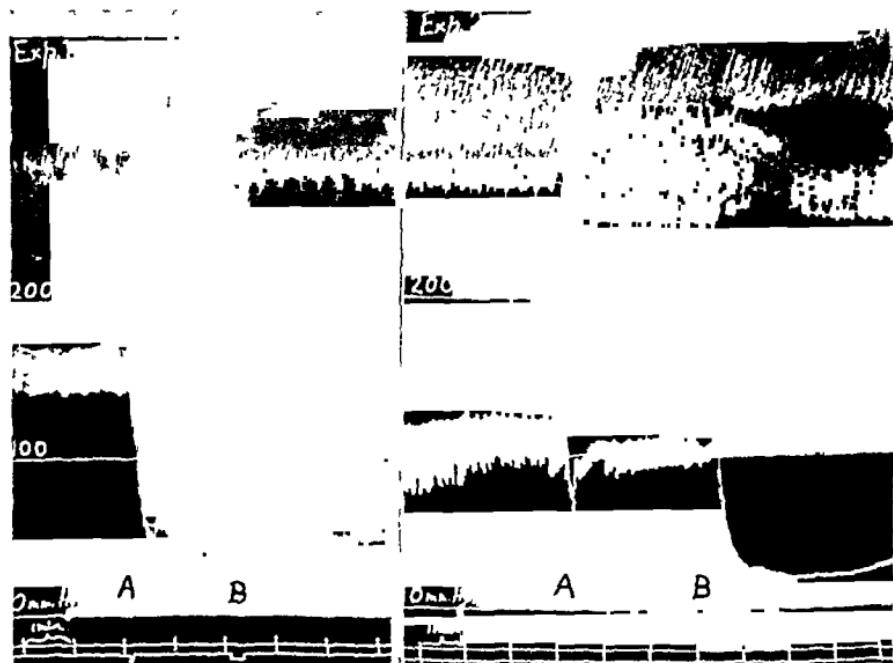


FIG. 4. Records of bronchioles (upper) and blood pressure (lower). *Exp. 1:* Dog, female, 13.7 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.36 mgm. per kgm.) intravenously B—lung distension with positive pressure (40 mm. Hg for 15 secs.). *Exp. 2:* Dog, male, 6.8 kgm. Pentothal sodium anesthesia. Vagi cut. A—lung distension with positive pressure (20 mm. Hg for 10 secs.). B—histamine (0.35 mgm. per kgm.) intravenously.

histamine bronchoconstriction considerably or even completely by the use of a sufficient degree of lung distension, and also to prevent the development of histamine bronchoconstriction by previously distending the lungs sufficiently. The two experiments in figure 4 show examples of these types of effects. Thus, in exp. 1, following histamine bronchoconstriction 15 seconds of lung distension with a positive pressure of 40 mm. of mercury almost completely antagonized the bronchoconstriction. There was little or no influence on the blood pressure level in this experiment. Again, in exp. 2, in the same figure, it is shown that distending the lungs prior to the injection of histamine almost completely pre-

vented the bronchoconstriction, although the usual fall in blood pressure still occurred. It is therefore clear that lung distension can both relieve and prevent histamine bronchoconstriction under proper conditions.

*2. Effects of lung distension on the responses to sprayed epinephrine and histamine.* Since the actions of epinephrine and histamine are upon the smooth muscle of the bronchioles, it was of interest to test the effects of lung distension upon the response to the spraying of these agents directly into the lungs, as described above. It was thought that in this way it might be possible to determine whether or not the intense blood pressure changes associated with injections of these agents were in any way concerned with the above-described effects.

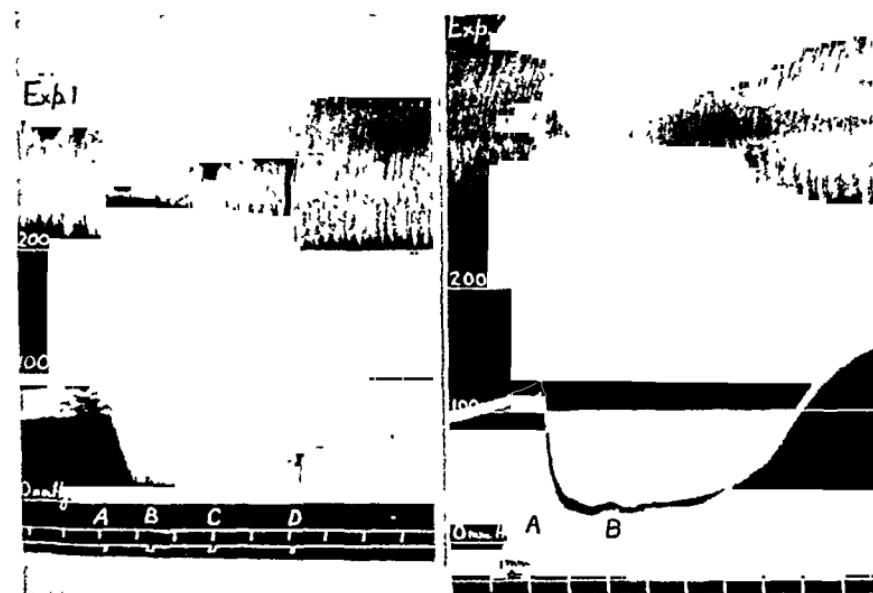


FIG. 5. Records of bronchioles (upper) and blood pressure (lower). *Exp. 1:* Dog, female, 5.0 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.7 mgm. per kgm.) intravenously. B and C—epinephrine (0.2 mgm.) sprayed (2 cc. 1 in 10,000 for 10 secs.). D—lung distension with positive pressure (40 mm Hg for 5 secs.). *Exp. 2:* Dog, male, 13.8 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (0.36 mgm. per kgm.) intravenously. B—epinephrine (1 mgm.) sprayed (1 cc. 1 in 1000 for 5 secs.).

In figure 5 (exp. 1) it may be seen that when the bronchioles were intensely constricted by an injection of histamine two successive sprayings of epinephrine induced moderate bronchodilatation, without any significant blood pressure changes—unlike the effects of injected epinephrine in similar dosages, as has been shown in the previous experiments. It might be noted that with the spraying of epinephrine, the second administration induced some further bronchodilatation, which was seldom seen after epinephrine injections. These bronchodilatations were submaximal and two minutes after the second epinephrine spray, distension of the lungs induced the very prompt and intense bronchodilatation shown, although no marked increase in blood pressure occurred. Similar results

were obtained in several other experiments. The spraying of a small dose of epinephrine can apparently, therefore, induce bronchodilatation without affecting the blood pressure and furthermore, this effect is potentiated by lung distension. It might be added that similarly spraying physiological saline induced no effect on the bronchioles.

In the other experiment shown (exp. 2, fig. 5), under similar conditions of histamine constriction the spraying of a very large dose of 1 mgm. of epinephrine induced a rapidly progressive bronchodilatation, associated with a slowly progressive increase in the blood pressure level. No lung distension was employed in this experiment but the large dose of epinephrine employed was sufficient to induce maximal bronchodilatation, without the usual acute pressor response to epinephrine.



FIG. 6. Records of bronchioles (upper) and blood pressure (lower). *Exp. 1:* Dog, female, 7.0 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (5.7 mgm. per kgm.) sprayed (2 cc. 2 per cent for 10 secs.). B and C—epinephrine (0.2 mgm.) intravenously. *Exp. 2:* Dog, female, 7.0 kgm. Pentothal sodium anesthesia. Vagi cut. A—histamine (2.85 mgm. per kgm.) sprayed (1 cc. 2 per cent for 5 secs.). B—epinephrine (0.2 mgm.) sprayed (2 cc. 1 in 10,000 for 10 secs.). C and D—epinephrine (0.2 mgm.) intravenously. E and F—lung distension with positive pressure (40 mm. Hg for 10 secs.).

Regarding the effects of spraying histamine, the results obtained were on the whole rather contrary to what was expected. In fact, in several experiments, the spraying of doses of histamine of 0.35 to 0.70 mgm. per kgm. (amounts which induce intense bronchoconstriction when given intravenously) led to little or no immediate bronchoconstriction, despite the fact that the blood pressure fell rapidly. Indeed, in order to obtain definite bronchoconstriction with histamine using the spray method, it was necessary to employ rather excessive doses. Thus, in figure 6 are shown results of two such experiments. In exp. 1 at A, 5.7 mgm. per kgm. of histamine were sprayed. There was a marked drop in blood pressure developing rapidly, but only a moderate degree of slowly developing bronchoconstriction. A somewhat similar effect is seen in exp. 2, at A, following the spraying of 2.85 mgm. per kgm. of histamine. Both of these

effects of histamine on the bronchioles contrast strikingly with the intense and almost immediate bronchoconstriction seen on the intravenous injection of much smaller doses of histamine as shown in figures 1, 2, 3 and 5.

Following histamine spraying two successive injections of epinephrine in exp. 1 (fig. 6), produced brief temporary pressor effects, and bronchodilatation. Despite this existing bronchodilatation the blood pressure continued to fall and the animal died within a few minutes. In exp. 2 in the same figure the spraying of epinephrine at B induced some bronchodilatation, but no effect on blood pressure. Four minutes later, however, the bronchioles were again markedly constricted. Two successive injections of epinephrine at C and D exerted only a slight bronchodilatation but the blood pressure was greatly improved. With distension of the lungs at E, there was however a good and sustained bronchodilatation, which could not be improved by a second period of lung distension at F. In this experiment again the bronchioles remained well dilated but the blood pressure continued to fall and the animal died despite the existing bronchodilatation. Death in both of these experiments was due to circulatory failure and not to bronchoconstriction.

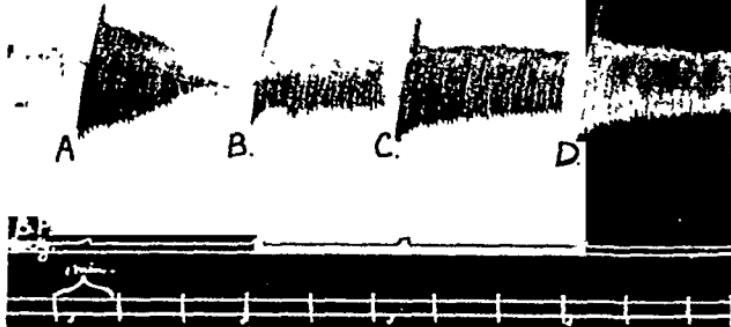
In connection with the effects of sprayed histamine on the bronchioles, it is of interest to note that Dautrebande and coworkers (2) have reported that following inhalation of small doses of histamine in man, as an aerosol of 1 per cent histamine solution, there was dilatation, rather than constriction, of the respiratory tract and an increase in the effective respiratory volume. Binet and Marquis (3) have also shown that histamine is destroyed during passage through the dog's lungs both *in vivo* and in isolated perfused ventilated lungs. This might explain the necessity of employing such excessive doses of histamine by the spray method. In view of the current interest in the antihistamine drugs and in view of the fact that histamine aerosols are so frequently employed experimentally in assessing the effects of these agents as possible anti-asthmatics, the problem of the effects of histamine by respiratory tract administration requires further study.

From the above results it appeared that these effects of lung distension might be primarily physical effects due to the mechanical stretching of the elastic tissue in the lungs. It was of interest therefore to test the influence of distending the lungs in similar fashion at the end of several experiments after the circulation of the animal had completely ceased. The results of two such experiments are shown in figure 7. Exp. 1, which is the termination of the experiment shown in figure 4 (exp. 2), shows that despite the absence of any circulation, the bronchioles were still somewhat dilated. Lung distension for 10 seconds with a pressure of 20 mm. of mercury at A, induced bronchodilatation lasting for 3 minutes. In this instance, the bronchioles at first dilated widely, then progressively closed down almost completely. Further repeated brief periods of lung distension at B, C, and D, produced similar but more prolonged bronchodilatation, despite the complete absence of the circulation.

Exp. 2 in the same figure, which was the termination of the experiment shown in figure 3 (exp. 1), shows at A, the effects of an intravenous injection of a large dose (50 mgm.) of histamine. Five minutes later, there was no evidence of any effective circulation, but the bronchioles were still intensely constricted. The

first brief lung distension at B induced only a slight transient bronchodilatation. Similar periods of lung distension at C and D led to prompt and sustained bronchodilatation. These results resemble rather closely those obtained in the animals with effective circulations, and would suggest that the effects of lung distension described above may be due primarily to the stretching of the elastic tissue of the lungs rather than to any effects on the smooth muscle or blood vessels of the lungs.

*Expt. 1.*



*Expt. 2.*

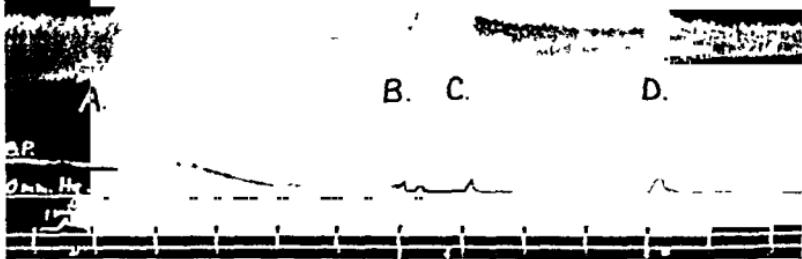


FIG. 7. *Expt. 1:* Termination of experiment 2 in fig. 4. A and D—lung distension for 10 secs. with positive pressure of 20 mm. Hg. B—lung distension for 10 secs. with positive pressure of 40 mm. Hg. C—lung distension for 15 secs. with positive pressure of 40 mm. of Hg. *Expt. 2:* Termination of experiment 1 in fig. 3. A—histamine (50 mgm.) intravenously. B and C—lung distension for 5 secs. with positive pressure of 40 mm. of Hg. D—lung distension for 10 secs. with positive pressure of 40 mm. Hg.

**DISCUSSION.** As is well known, the lung is extremely rich in elastic tissue, which runs lengthwise along the bronchioles and extends beyond the muscle fibres to the alveoli (4). The elastic tissue is responsible physiologically for the normal recoil of the lungs after inspiration, that is, during expiration. It has been shown by Ellis and Livingston (5) and Ellis (6) that the bronchial tree mechanically dilates during inspiration and constricts during expiration. It is conceivable, therefore, that during the intense smooth muscle bronchoconstriction induced by histamine, the normal mechanical dilatation during

inspiration is prevented. It is possible, then, that although the epinephrine relaxes the smooth muscle of the bronchioles, so that they dilate somewhat, this may not be sufficient to overcome this existing mechanical disadvantage. Under such conditions, therefore, the bronchodilator effect of epinephrine is submaximal, and cannot be further augmented by further injections of epinephrine, that is to say, a "refractory state" has developed. Sudden distension or stretching of the elastic tissue might then overcome this obstruction to bronchodilatation, and thus initiate the normal functioning of the elastic tissue. The stretched elastic tissue may in turn exert a favorable influence on the smooth muscle relaxation. Ellis (6) believes that the smooth muscle of the bronchioles is constantly in a state of tone controlled by sympathetic and parasympathetic nerves. When the tone is lowered by epinephrine (bronchodilatation) the stretching of the elastic tissue might then further augment this effect, and lead to the observed potentiated responses.

In conclusion, the above described experiments would seem to indicate that the use of lung distension by positive pressure under suitable conditions might be a valuable aid in the relief of intense asthmatic attacks and particularly in the condition of status asthmaticus, where the bronchioles are refractory to injected epinephrine.

#### SUMMARY

Dogs deeply anesthetized with pentothal sodium and vagotomized, were used. The changes in the bronchioles (Jackson Method) and the blood pressure were recorded simultaneously.

It is shown that after histamine bronchoconstriction and fall in blood pressure, repeated injections of epinephrine induced only submaximal bronchodilatation, and often complete refractoriness of the bronchioles to epinephrine was observed, despite the fact that the blood pressure was still responsive. At this time, a brief period (5 to 10 seconds) of lung distension with positive pressure (40 mm. of mercury) induced marked and sustained maximal bronchodilatation.

The bronchoconstrictor action of histamine under these conditions may also be partially or completely antagonized by lung distension with positive pressure.

It is suggested that these effects of lung distension are due primarily to mechanical stretching of the elastic tissue of the lungs.

It is also shown that epinephrine is a more effective bronchodilator agent when sprayed directly into the lungs, than when injected; and conversely, histamine is a less effective bronchoconstrictor agent when sprayed than when injected.

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# THE COMPARATIVE PHARMACOLOGY OF p-FLUOROPHENETHYL-AMINE AND p-FLUOROPHENETHYL-METHYLAMINE

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Since the fundamental study of Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. Only a few studies have been concerned with the influence of introduction of halogen atoms in the benzene ring of the basic phenethylamine structure (2-4). Suter and Weston (5) have recently synthesized p-fluorophenethylamine and p-fluorophenethyl-methylamine.<sup>1</sup> In this study, these compounds<sup>2</sup> have been compared with the corresponding unsubstituted phenethylamine and phenethyl-methylamine and with the p-hydroxyphenethylamine, tyramine.

**BLOOD PRESSURE EFFECTS.** *1. Method.* Hjort (6) has pointed out that repeated doses<sup>3</sup> of the phenethylamines and phenethyl-methylamines may be given at frequent intervals without significant change in quantitative response. Consequently, the five agents under test were given in rotation to a comparatively small number of animals.

**Dogs.** Ten apparently healthy, adult mongrel dogs (7-21 kgm.) were used; these were anesthetized with sodium barbital (330 mgm./kgm.) administered intraperitoneally 90 minutes prior to use. One mgm. of atropine sulfate per kgm. was administered intravenously prior to the arterial cannulation. Carotid arterial blood pressure was recorded with the usual mercury manometer and soot kymograph. Five of the animals were standardized with graded doses of epinephrine (4-20 microgm./kgm.) and one mgm. test doses of the agents per kgm., as 1 per cent solutions, injected into a femoral vein. The other five animals were standardized with graded doses of epinephrine (1-12 microgm./kgm.) and one-half mgm. test doses per kgm. used. See fig. 1 for portions of a typical experiment.

**Cats.** Since much of the earlier work with sympathomimetic amines has been done with the cat, we prepared 7 cats in the same manner as the dogs except that the median circumflex branch of the femoral artery was subcannulated and the pressure changes recorded with a Lambert-Wood (7) strain-gage manometer recording through a Heiland type A galvanometer on photographic paper. The customary standardization with epinephrine was performed and the agents under investigation injected every 15 minutes for five to ten injections.

**Rabbits.** Four rabbits were prepared in the same manner as the cats, except that only 270 mgm. of sodium barbital per kgm. was used as an anesthetic. Tyramine and epinephrine yield reproducible results in a given rabbit; the other agents do not. Changing the dose from 0.5 to 4 mgm./kgm. sometimes increased the pressor effect, sometimes produced

<sup>1</sup> In accordance with established usage, these compounds are named as derivatives of "phenethylamine" or "beta-phenethylamine" instead of through the systematic chemical name 2-phenyl-ethylamine.

<sup>2</sup> I am grateful to Dr. C. M. Suter, Winthrop-Stearns, Inc., Rensselaer, N. Y., for generously supplying the fluorocompounds. I am grateful to D. A. Herring for technical assistance.

a depressor effect, sometimes no effect. This difficulty with rabbits has been observed previously (8, 9). This portion of the problem was abandoned.

*Rats and guinea pigs.* Eight rats (0.22–0.31 kgm.) and eight guinea pigs (0.40–0.75 kgm.) were prepared in the same manner as the cats, except that the carotid artery was cannulated and only 250 mgm. of sodium barbital per kgm. was used as anesthetic. It was found necessary to use doses of the agents of the order of 2 mgm./kgm. to obtain reasonable rises in blood pressure. Portions of a typical record are reproduced in fig. 2.

*2. Results.* The results of the epinephrine-agent equivalence are summarized in the table. The use of a fixed dose of 1 mgm. of agent per kgm. is comparable

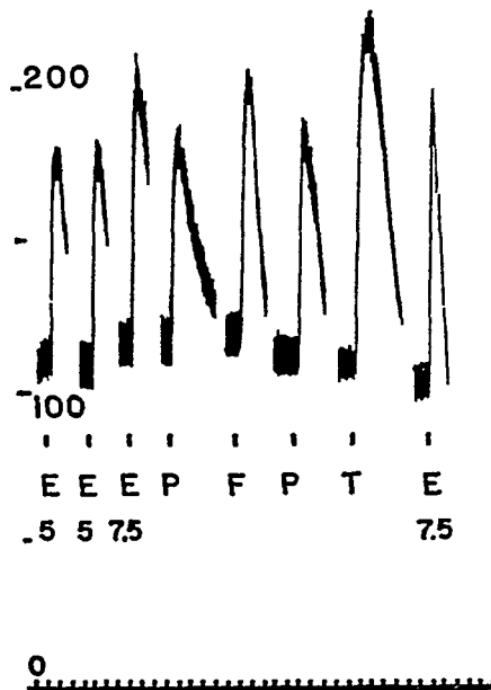


FIG. 1. Dog (female; 10 kgm.). 330 mgm. of Na barbital per kgm. 1 mgm. of atropine sulfate per kgm. Blood pressure in mm. Hg, ordinate; time, in minutes, abscissa. Time interval between injections, 15 minutes. E = l-epinephrine (as hydrochloride) in microgm./kgm. as indicated. Five-tenths mgm. other drugs per kgm.: P = phenethylamine hydrochloride, F = p-fluorophenethylamine hydrochloride, and T = tyramine hydrochloride.

to the work of others in the field. However, this dose of p-fluorophenethylamine and tyramine produce tremendous rises in mean blood pressure, and the difference in blood pressure rise per microgram increment of epinephrine is very small at these levels. Consequently, a comparable group of experiments at 0.5 mgm./kgm. was carried out. To comply with the dictum of Barger and Dale (1) that sympathomimetic agents should be compared on equimolecular bases, the data available have been expressed in terms of moles of amine base and compared with the pressor equivalent number of moles of epinephrine base. These activity ratios are given in the table. The relative results obtained at

the 0.5 mgm. and 1.0 mgm. dose level are reasonably similar for the number of experimental observations involved.

The average blood pressure rise following 5 microgm. of epinephrine per kgm. was 77.1 mm. Hg for the dogs, 41.5 for the cats, 51 for the rabbits, 71.4 for the rats, and 80.4 for the guinea pigs.

**PERFUSED HEART.** Since these agents produce cardioacceleration and perhaps increased cardiac output, 4 rabbit hearts and 3 cat hearts were prepared for perfusion by the method of Langendorff, and rate, force of contraction, and coronary outflow were recorded. Test doses of epinephrine and the agents under

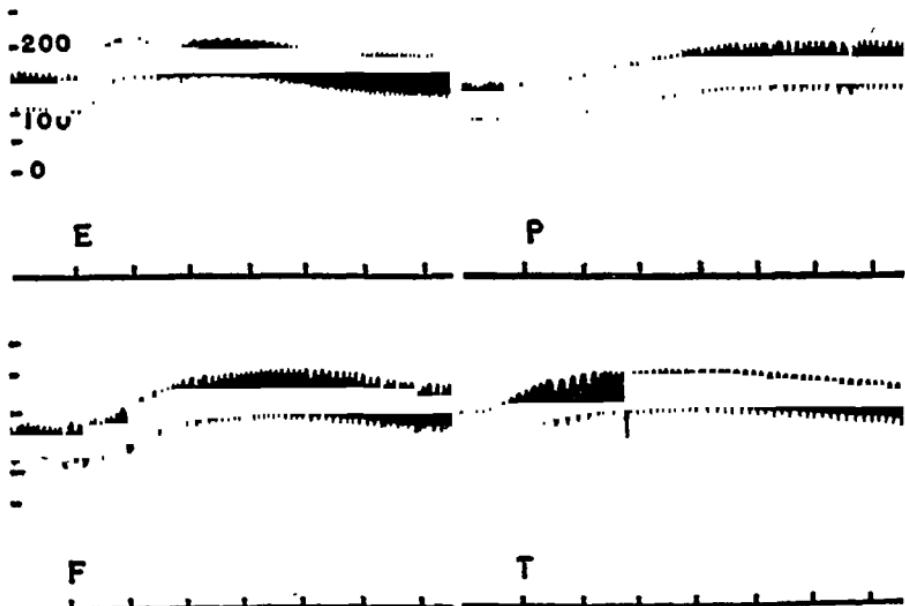


FIG. 2. Rat (male; 0.27 kgm.). 250 mgm. of Na barbital per kgm. 1 mgm. of atropine sulfate per kgm. Blood pressure in mm. Hg, ordinate; time, in 10 second intervals, abscissa. 8.75 minutes of record deleted between injections. E = 5 microgm. 1-epinephrine per kgm., P = 2 mgm. phenethylamine hydrochloride per kgm., F = 2 mgm. p-fluorophenethylamine hydrochloride per kgm., and T = 2 mgm. tyramine hydrochloride per kgm.

investigation were added alternately to the perfusion fluid. One mgm. epinephrine per liter of perfusion fluid doubled the activity of the rabbit hearts and often tripled the output of the cat hearts. Ten to fifty times this dose of tyramine had comparable effects. One cat heart doubled rate and force of contraction at 20 mgm. p-fluorophenethylamine per liter; lower and higher concentrations had less effect. All the other preparations exhibited depression from this agent and the other phenethylamines. Similar results for tyramine (10) and phenethylamine (8, 11) have been observed previously.

**ACTION ON BRONCHI.** None of these agents gave evidence of bronchodilation in histamine-constricted guinea pig lungs prepared by the method of Tainter,

Pedden, and James (12), although good bronchodilation by epinephrine was obtained. Apparently the simple sympathomimetic agents closely related to phenethylamine are unable to produce effective bronchodilation or antagonism of histamine.

**ACTION ON ISOLATED RABBIT JEJUNUM.** Three sections of jejunum from each of 5 rabbits were placed in oxygenated Tyrode solution. Concentrations of the five agents of less than  $1 \times 10^{-3}$  Molar (about 160-190 microgm./ml.) had little

TABLE I

CHEMICAL NAME (AMINE HYDROCHLORIDES) AND FORMULA	VASOPRESSOR ACTIVITY					
	Micro- grams epine- phrine equivalent to one-half m.m.g. amine hydro- chloride	Micro- grams epine- phrine equivalent to one m.m.g. amine HCl	Molecules of amine equivalent in pressor effect to one molecule of l-epine- phrine	Micro- grams epine- phrine equiva- lent to one m.m.g. amine HCl	Micrograms epinephrine equivalent to two m.m.g. amine hydrochloride	
	Dog	Dog	Dog	Cat	Rat	Guinea pig
Phenethylamine 	5.0	10.5	116-111	7.8	4.0	5.0
Phenethyl-methylamine 	3.6	7.9	148-135	5.7	3.0	2.2
p-Fluorophenethylamine 	7.0	13.1	74-80	8.4	5.0	5.5
p-Fluorophenethyl-methylamine 	5.0	9.6	97-100	4.5	3.5	4.0
Tyramine 	10.0	16.0	53-66	10.0	5.5	7.5

All blood pressure rises obtained are within the range of  $\pm$  one microgram of the epinephrine equivalence given, except for tyramine at one m.m.g./kgm. and here the range is  $\pm$  three micrograms.

or no action. Phenethylamine in a concentration of  $1 \times 10^{-3}$  Molar had a slight contractile action on all the strips, the phenethyl-methylamine had a slightly greater effect in six and less in the other nine, the p-fluorophenethylamine had considerably greater effect in all, but tyramine had the most contractile activity of any of the agents. The p-fluorophenethyl-methylamine activity was between phenethylamine and p-fluorophenethylamine. Higher concentrations of these agents often produced diminution of tone, but the segments seldom returned to normal after "washing out" the amine.

**DISCUSSION.** Barger (2) noted that p-chlorophenethylamine was more potent as a vasopressor agent than the unsubstituted compound, although less active than tyramine. This investigation has shown that the introduction of the fluorine atom into the para position of the phenethylamine molecule also leads to an increase in activity. If the phenethylamine type agents simply act as ions that are able to stimulate vascular sympathetic nerve endings (13), then the substitution of relatively electronegative atoms (14) like fluorine and chlorine, in the para position of the benzene ring, might be expected to influence the ionic strength of the molecule or the potential coulomb forces of attraction between the ion and the acceptor substance. This scarcely explains the greater activity of the para hydroxy compound or why these agents act more or less in a cholinergic manner on some isolated tissues.

#### SUMMARY

The replacement of the hydrogen atom in the para position of phenethylamine and phenethyl-methylamine with a fluorine atom leads to compounds that are more active as sympathomimetic amines, although not as active as tyramine, the compound with a hydroxy group in the para position of the phenethylamine molecule.

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# PHARMACOLOGY OF A NEW ANTIHISTAMINE, $\beta$ -PYRROLIDINE-ETHYL-PHENOTHIAZINE (PYRROLAZOTE)

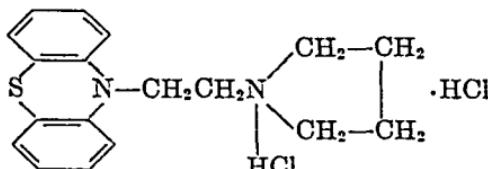
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Modern progress in the development of drugs possessing specifically high antihistaminic properties is due in large measure to studies with the Fourneau series of compounds, which showed that dialkyl amino ethyl ethers (1) and ethylenediamines (2) were active in preventing histamine responses. Although such compounds were too toxic to be of practical value, the search by the French investigators for other synthetic compounds having a higher degree of activity and lower toxicity was rewarded with the discovery of these useful compounds, N<sup>1</sup>-phenyl-N<sup>1</sup>-benzyl-N, N-dimethyl ethylenediamine (Antergan, 2339 RP) and N<sup>1</sup>-p methoxybenzyl-N<sup>1</sup>- $\alpha$  pyridyl-N, N-dimethyl ethylenediamine (Neoantergan, 2786 RR) (3). Similar accomplishment of high activity and low toxicity was obtained in this country with the discovery of several compounds, early examples of which are  $\beta$ -dimethylaminoethyl benzhydryl ether, Benadryl, (4) and N<sup>1</sup>- $\alpha$  pyridyl-N<sup>1</sup>-benzyl-N, N-dimethyl ethylenediamine, Pyribenzamine, (5).

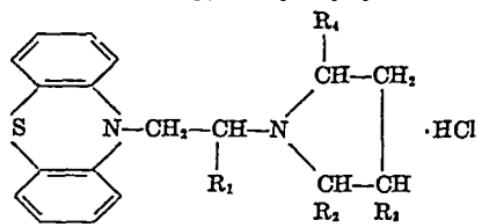
In attempting to contribute further to the development of compounds with high antihistaminic activity and low toxicity, we have investigated a large series of compounds. During the course of these studies it was observed that replacement of the dimethyl amino radical of Benadryl with the pyrrolidine grouping resulted in enhancement of both local anesthetic and antihistaminic properties. Coincident with these studies, there appeared a report by Halpern (6) that certain thiadiphenylamine derivatives were of higher antihistaminic potency than Neoantergan. Several pyrrolidyl-alkyl-phenothiazines were therefore prepared (7). In evaluating the antihistaminic potencies and the toxicities of the members of this group of compounds thus far studied, one of them was found to be outstanding, because of its high activity and low toxicity. This compound is  $\beta$ -pyrrolidine-ethyl-phenothiazine mono-hydrochloride (I-WBR-86), for which the name Pyrrolazote has been chosen. Its structural formula is as follows:



The pharmacologic properties of this compound were studied in further detail and compared with those of N<sup>1</sup>- $\alpha$  pyridyl-N<sup>1</sup>-benzyl-N,N-dimethyl ethylenediamine mono-hydrochloride (Pyribenzamine hydrochloride) in parallel experiments.

**ANTAGONISM OF SOME OF THE PHARMACOLOGIC AND TOXICOLOGIC ACTIONS OF HISTAMINE.** Compounds were screened for antihistaminic activity by the isolated smooth-muscle test (method of Magnus). Evaluation of the activity in blocking the spasmogenic effect of histamine was made on isolated segments of guinea pig small intestine. The muscle segments, in a constant temperature bath at 37.5°C., were attached to a lever recording on a kymograph. In each experiment several equal responses of the muscle to 1.0 microgram of histamine dihydrochloride per cc. of Tyrode's solution bathing it were first recorded. The comparative activities of the pyrrolidylalkyl-phenothiazines are given in table 1. It is apparent that compound I-WBR-86 is the most active of the group. A direct comparison of this compound with Pyribenzamine by the isolated smooth-muscle test is shown in figure 1. The recordings of two successive responses of histamine are given prior to the introduction of the antihistamine. I-WBR-86

TABLE 1

*Antihistaminic activity of some N-pyrrolidylalkyl phenothiazine hydrochlorides*

R	R <sub>1</sub>	R <sub>2</sub>	R <sub>4</sub>	CODE NO.	ACTIVITY*
H	H	H	H	I-WBR-86	3 to 4
H	H	CH <sub>3</sub>	CH <sub>3</sub>	I-WBR-127	3/4
H	CH <sub>3</sub>	H	CH <sub>3</sub>	I-WBR-138	1/4
CH <sub>3</sub>	H	H	H	JBW-149-VII	1/5
CH <sub>3</sub>	H	CH <sub>3</sub>	CH <sub>3</sub>	I-WBR-126A	1/4
CH <sub>3</sub>	CH <sub>3</sub>	H	CH <sub>3</sub>	JBW-II-14	1/3

\* Benadryl = 1.

was then added two minutes before the third administration of the same amount of histamine, and an inhibition of the response thus demonstrated. Subsequent doses of histamine, all of which were given at ten-minute intervals, did not elicit a normal response until the eighth dose after I-WBR-86, or seventy-two minutes later. The muscle, then having attained normal responsiveness, was used to determine the effect of Pyribenzamine. It can be seen that the degree of inhibition to histamine effected by this drug is practically identical with that obtained with I-WBR-86. The duration of effect of Pyribenzamine by this test is, however, only one-eighth that of I-WBR-86, since the same dose of histamine twelve minutes after Pyribenzamine again elicited a normal response. By reversing the order of administration of the drugs in other experiments, the same difference in duration of effect was apparent.

The atropine-like properties of I-WBR-86 were investigated using as test

objects isolated segments of rabbit intestine contracted by the addition of acetylcholine chloride to the bath fluid. It was found that between two hundred and fifty and five hundred times as much I-WBR-86 as atropine sulfate was required to obtain the same degree of relaxation. Published experiments (8)

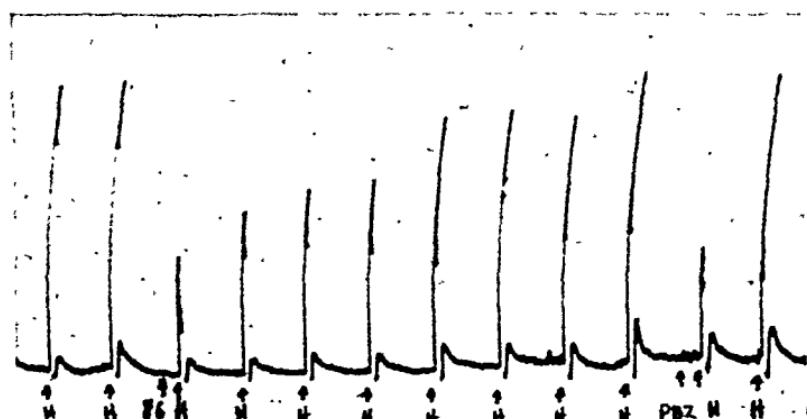


FIG. 1. RESPONSES OF AN ISOLATED SEGMENT OF GUINEA PIG ILEUM TO 1.0 MICROGRAM OF HISTAMINE DIHYDROCHLORIDE PER CC. AND THE EFFECTS OF 0.2 MICROGRAMS OF PYRROLAZOTE (I-WBR-86) AND PYRIBENZAMINE PER CC. THEREON

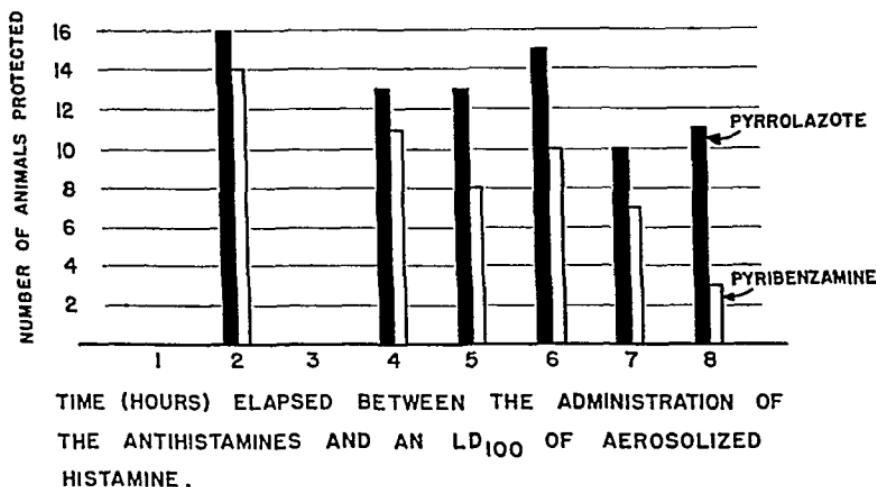


FIG. 2. PROTECTION AFFORDED GUINEA PIGS BY PYRROLAZOTE (I-WBR-86) AND PYRIBENZAMINE (2.0 MGm./KGM. I.P.) AGAINST AN L.D.<sub>100</sub> OF AEROSOLIZED HISTAMINE Sixteen animals in each group. None of sixteen untreated guinea pigs survived

indicate that while Benadryl is more active than this, possessing approximately one-fiftieth the activity of atropine sulfate, Pyribenzamine is practically devoid of this type of activity. These studies indicate that I-WBR-86 is highly specific as a histamine antagonist.

*Intoxication of guinea pigs.* The protection afforded by the antihistamines to guinea pigs exposed to an aerosol of histamine in a closed chamber has been used in these laboratories to evaluate their effectiveness. The method employed was that described by Loew and co-workers (4). Prior to the testing of I-WBR-86 it had been found that Pyribenzamine afforded as great or greater protection than any other substance. I-WBR-86 and Pyribenzamine were therefore run in parallel on paired groups of guinea pigs until the series reported in figure 2 was completed. The amount of histamine introduced into the chamber by aerosol at a constant rate which would kill all of the unprotected animals was determined. The protection afforded by 2.0 mgm. of antihistamine per kgm. and the duration of this effect were then determined by exposing the treated guinea pigs at varying periods of time to a normally fatal dose of histamine. It is apparent from the results in figure 2 that the protection afforded up to four hours after injection is comparable for both drugs. At the four remaining time intervals, however, I-WBR-86 definitely protects more animals than does Pyribenzamine. The greatest difference in protection occurred eight hours after

TABLE 2

*An estimation of the protection afforded guinea pigs by I-WBR-86 and Pyribenzamine against histaminic intoxication as measured by their increased tolerance to histamine*

DOSE OF HISTAMINE DIHYDROCHLORIDE INTRACARDIALLY <i>mgm. per kgm.</i>	NO. OF LETHAL DOSES	RATIO OF NUMBER OF GUINEA PIGS SURVIVING TO NUMBER OF GUINEA PIGS USED		
		Controls	I-WBR-86	Pyribenzamine
			1.0 mgm. per kgm. s.c. 15 min. prior to histamine	
0.6	1	0/6		
12.0	20		6/10	3/3
18.0	30		6/13	8/13

the administration of the antihistamines, at which time sixty-nine per cent of the guinea pigs on I-WBR-86 survived whereas only nineteen per cent of those on Pyribenzamine survived. I-WBR-86, therefore, appears to be effective for a longer period of time than Pyribenzamine as judged by both the activity it exhibits against histamine spasms of smooth muscle *in vitro* and the protection it affords against fatal histamine intoxication by aerosol *in vivo*.

The antihistaminic activity of I-WBR-86 was demonstrated also against histamine introduced directly into the blood stream, either by vein or intracardially. In normal guinea pigs not protected by an antihistamine, 0.6 mgm. of histamine dihydrochloride per kgm. caused death in one hundred per cent of the animals. This toxicity of histamine in guinea pigs can be decreased several fold by the prior administration of I-WBR-86. The results which are recorded in table 2 show that 1.0 mgm. of either I-WBR-86 or Pyribenzamine per kgm. given subcutaneously fifteen minutes before the histamine protected approximately fifty per cent of those guinea pigs administered thirty times the LD<sub>100</sub> of histamine intracardially.

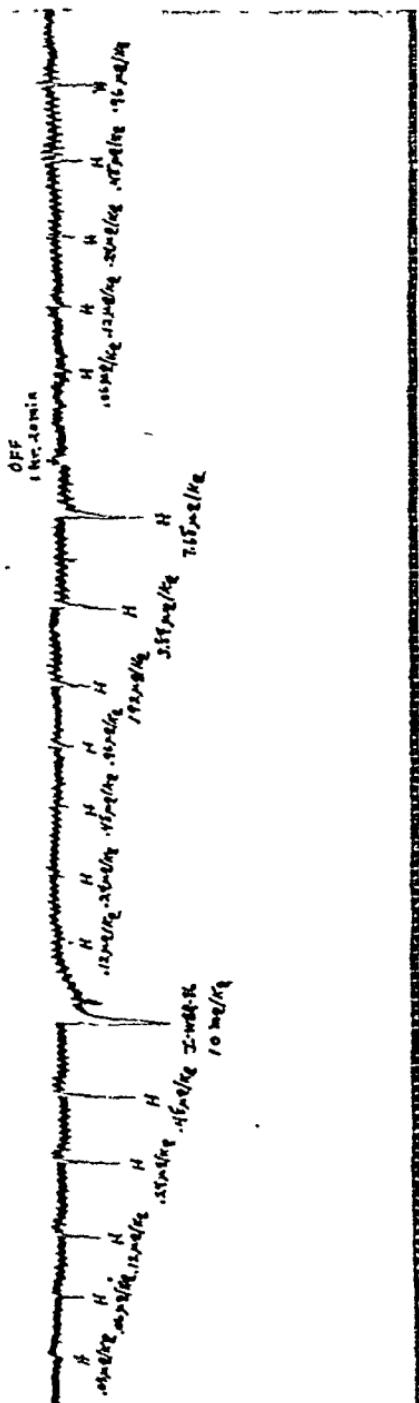


FIG. 3. CAROTID BLOOD PRESSURE TRACING OF A CAT ANESTHETIZED WITH SODIUM PHENOBARBITAL. Pyrrolazolo (I WBR-86) by vein at 1.0 mgm per kgm produced a transient vasodepression of 81 mm. Hg while raising (the threshold to histamine (II), as measured by depressor responses, approximately sixteen fold. Time in minutes.

The minimum dose of these antihistamines effective in affording protection against one LD<sub>100</sub> of histamine was also determined and found to be approximately 20 micrograms per kgm. In the series of guinea pigs used in this experiment, a summary of which is given in table 3, 0.6 mgm. of histamine dihydrochloride per kgm. was found to be the minimum dose killing all of a group of five. Thirteen of a group of twenty, however, survived the same dose of histamine, after having been given only 20 micrograms of I-WBR-86 subcutaneously

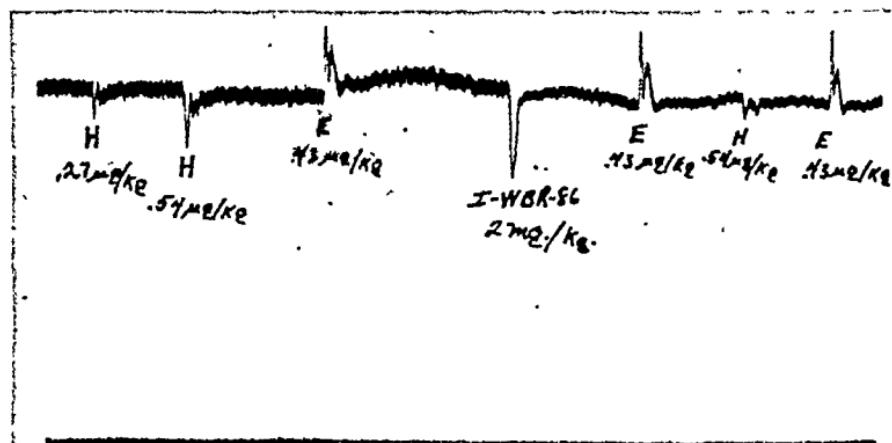


FIG. 4. CAROTID BLOOD PRESSURE TRACING OF A DOG ANESTHETIZED WITH SODIUM PHENOBARBITAL

Pyrrolazote (I-WBR-86) by vein at 20 mgm. per kgm. produced a transient vasodepression of 58 mm. Hg. It had no effect on the pressor response to epinephrine (E) while inhibiting the depressor response to histamine (H) 69 per cent. Time in minutes.

TABLE 3

*An estimation of the M E.D. of I-WBR-86 and Pyribenzamine in affording protection to one L.D. <sub>100</sub> of histamine dihydrochloride by vein (0.6 mgm./kgm.)*

DOSE OF ANTIHISTAMINE micrograms per kgm	RATIO OF NUMBER OF GUINEA PIGS SURVIVING TO NUMBER OF GUINEA PIGS USED		
	Controls	I-WBR-86	Pyribenzamine
0.0	0/5		
10.0			2/10
20.0		13/20	8/10

fifteen minutes before the histamine. Again, the protection afforded was found to be comparable to that given by Pyribenzamine.

*Vasodepression in cats and dogs.* Histamine vasodepression in anesthetized cats and dogs can be effectively antagonized by I-WBR-86 administered either parenterally or orally. Reproductions of kymograph tracings of a few experiments are given in figures 3, 4, 5 and 6. From the responses recorded in figure 3 it is apparent that I-WBR-86 at a dose of 1.0 mgm. per kgm. in this cat raised the threshold to histamine approximately sixteen-fold, 3.84 and 7.68 micrograms of histamine per kgm. being necessary after the drug to obtain responses com-

parable to those produced prior to it by 0.24 and 0.48 micrograms per kgm., respectively. The period of time that elapsed between I-WBR-86 administration and the last injection of histamine was three hours, and although the histamine antagonism at this time was beginning to lessen there was still an eight-fold increase in the threshold to histamine, since 0.96 micrograms per kgm. produced about the same vasodepression as 0.12 micrograms per kgm. did before the anti-histamine.

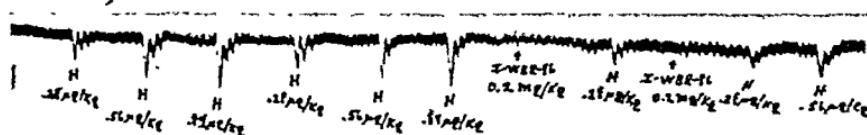


FIG. 5. CAROTID BLOOD PRESSURE TRACING OF A DOG ANESTHETIZED WITH SODIUM PHENOBARBITAL

Pyrrolazote (I-WBR-86) intravenously in small doses (0.2 mgm./kgm.) produced no vasodepression while inhibiting the depressor response to histamine (H) from 40 to 60 per cent. Time in minutes.

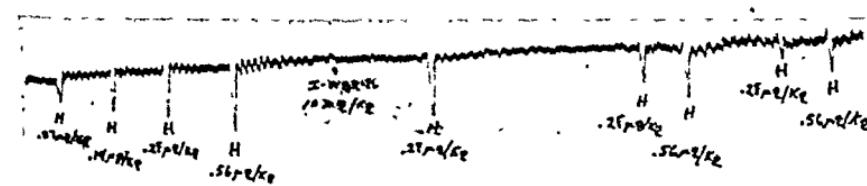


FIG. 6. CAROTID BLOOD PRESSURE TRACING OF A CAT ANESTHETIZED WITH SODIUM PHENOBARBITAL

Pyrrolazote (I-WBR-86) by stomach tube at 10 mgm./kgm. raised the threshold to histamine (H) approximately four times. Time in minutes.

Antagonism to histaminic vasodepression in a dog is shown in figure 4, where 2.0 mgm. of I-WBR-86 per kgm. reduced the response to 0.54 micrograms of histamine per kgm. approximately seventy per cent. From figure 5 it can be seen that a dose as low as 0.2 mgm. per kgm. inhibited the depressor response to histamine in a second dog nearly forty per cent. These tracings, in addition to demonstrating the marked antihistaminic activity of I-WBR-86, show that doses of 1.0 mgm. per kgm. and greater by vein produced an immediate but transient fall in blood pressure in these species. I-WBR-86 in smaller amounts did not produce vasodepression and was still very active as shown by the results obtained by 0.2 mgm. per kgm. in the second dog mentioned above.

The effectiveness of I-WBR-86 after oral administration to a cat is illustrated

in figure 6. Maximum inhibition of histaminic vasodepression was not apparent until about one hour after 10 mgm. per kgm. had been given by stomach tube, at which time approximately a four-fold increase in the threshold to histamine was attained.

In several experiments with both cats and dogs, it was observed that I-WBR-86 had no effect on the pressor responses to epinephrine. Typical responses are recorded in figure 4 where the pressor response to 0.43 micrograms of epinephrine per kgm. remained unchanged after 2.0 mgm. of I-WBR-86 per kgm. The fact that the pressor response to epinephrine was not affected differentiates I-WBR-86

TABLE 4  
*Determination of the L.D.<sub>100</sub> of egg white in sensitized guinea pigs*

AMOUNT INJECTED	DILUTION	SURVIVAL RATIO
cc.		
0.1	1-8	0/3
0.1	1-12	0/9
0.1	1-16	1/3
0.1	1-24	1/5

TABLE 5

*An estimation of the protection afforded shocked guinea pigs by the prior administration of antihistamines*

Animals sensitized to hen egg white; shocking dose 0.1 cc. of 1:12 diln.

DOSE	SURVIVAL RATIO	
	I-WBR-86	Pyribenzamine
mgm. per kgm.		
1.0	1/3	2/3
2.0	2/3	2/3
3.0	2/3	2/3
4.0	3/6	3/6
8.0	7/10	8/10
16.0	3/6	2/6
32.0	3/3	3/3

in this respect from several other antihistamines. Sherrod, Loew and Schloemer (9) reported that Benadryl, Pyribenzamine and Neoantergan all enhanced the pressor response to epinephrine. Yonkman and co-workers (10) had also observed this effect with Pyribenzamine. More recently enhancement of the pressor activity of epinephrine was observed also for N<sup>1</sup>(2-pyridyl)-N<sup>1</sup>(2-phenyl)-N, N-dimethyl ethylenediamine hydrochloride by Lee, Dinwiddie and Chen (9).

ANTIANAPHYLACTIC PROPERTIES. Conflicting results have been reported in the literature concerning the action of the antihistamines in affording protection against anaphylaxis. Benadryl and Pyribenzamine have been investigated with regard to action on true anaphylactic shock in guinea pigs. That these sub-

stances do afford protection against anaphylaxis is indicated by the results obtained in several laboratories (5, 12, 13). Campbell and associates (14) have reported, however, that although Benadryl was effective against histaminic shock in rabbits no apparent protection was afforded sensitized rabbits and guinea pigs against a shocking dose of antigen (hen egg white).

To determine the antianaphylactic properties of I-WBR-86, guinea pigs actively sensitized to hen egg white were used. The pigs were sensitized by two intraperitoneal injections of 0.1 cc. of undiluted egg white four days apart. Approximately three weeks later the minimum intracardial dose of egg white which killed one hundred per cent of unprotected pigs was determined (see table 4). This amount, 0.1 cc. of a 1:12 dilution of egg white, was then used

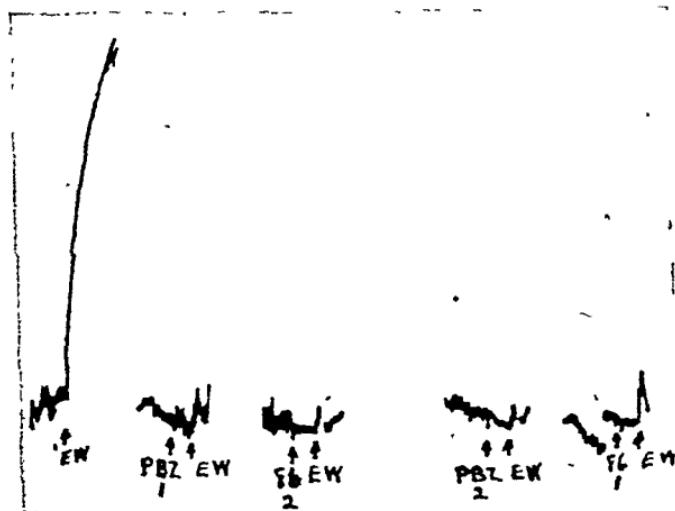


FIG. 7. RESPONSES OF ISOLATED SEGMENTS OF THE ILEUM OF A SENSITIZED GUINEA PIG TO EGG WHITE 1:12,500 BOTH IN THE ABSENCE AND PRESENCE OF PYRROLAZOTE (I-WBR-86) AND PYRIBENZAMINE

The antihistamines were present at concentrations of 0.2 and 0.4 micrograms per cc. at 1 and 2, respectively.

as the challenging dose of antigen administered intracardially fifteen minutes after a subcutaneous dose of the antihistamine. Table 5 shows that I-WBR-86 and Pyribenzamine afforded comparable protection against anaphylaxis. One hundred per cent survival in this series was attained only at the 32 mgm. per kgm. dose, however. In a second series not run in parallel with Pyribenzamine and on pigs from a different source, I-WBR-86 at 2.5 and 5.0 mgm. per kgm. gave complete protection to two groups of five pigs challenged with a dose of antigen which killed fourteen of fifteen untreated animals.

To demonstrate further that I-WBR-86 exerts an antianaphylactic effect, segments of small intestine from guinea pigs sensitized to egg white were used for studies *in vitro*. The muscle bath and technique used for recording muscular spasms were the same as were used to record inhibitions of histaminic spasms.

Intestinal segments from sensitized animals contracted in the presence of egg white 1:12,500 (Schultz-Dale Phenomenon), whereas segments from non-sensitized animals did not. When I-WBR-86 was added to the Tyrode's solution bathing the intestine at a concentration of 0.4 micrograms per cc., the effect of a subsequent addition of egg white was almost completely antagonized. For purposes of comparison Pyribenzamine was run in parallel. The tracing re-

TABLE 6  
*Acute toxicity*

DOSE <i>mgm./kgm.</i>	MOUSE				RAT		RABBIT	
	Intravenous		Subcutaneous		Intravenous		Intravenous	
	I-WBR-86	PBZ	I-WBR-86	PBZ	I-WBR-86	PBZ	I-WBR-86	PBZ
10							2/10	
12							2/10	
14			1/10					
15			3/10				4/10	
16			7/10				4/10	
18			6/10				4/10	
20			7/10				10/10	
23								
24					4/10			
27					6/10		2/5	
30	0/3				7/10			
32							1/5	
33	4/10							
36	3/10							
38	6/10						3/5	
40	6/6							
45							3/5	
50					1/9			
62					4/10			
75					9/9			
800			5/20					
1000			7/20					
1200			5/20					
1500			10/20					
1800			15/20					
Approx. LD <sub>50</sub> . . . . .	37	16	1340	64	26	16	36	12

corded in figure 7 shows that I-WBR-86 and Pyribenzamine were equally effective in preventing this type of response.

**TOXIC ACTION AND OTHER PROPERTIES.** *Acute toxicity.* Estimates were made of the amounts of both I-WBR-86 and Pyribenzamine required to produce mortality in mice, rats and rabbits. The results tabulated in table 6 show that I-WBR-86 is considerably less toxic than Pyribenzamine in these species. Symptoms of intoxication at high doses consisted of convulsive seizures and rapid

respiration preceded in some instances by prostration and paralysis of short duration.

*Chronic toxicity.* Young white male rats (60 grams) in groups of ten were administered I-WBR-86 by stomach tube five days each week at five dosage levels: namely 10, 25, 75, 150 and 300 mgm. per kgm. for periods of 10, 10, 10, 8½ and 4½ weeks respectively. Control groups of rats were administered tap water. Symptoms of intoxication were observed in rats receiving doses greater than 75 mgm. per kgm. Drowsiness, ruffled coats and difficult breathing were the chief symptoms. Those rats on 300 mgm. per kgm. developed distended abdomens which were caused by enlargement of the liver. A dose of 10 mgm. per kgm. produced no gross pathology, and growth was not impaired. Rats on larger amounts gained less than untreated controls.

An extensive histopathologic study of the tissues of these animals by Dr. A. James French, Associate Professor of Pathology at the University of Michigan, revealed no changes from the normal in any organ except for degenerative fatty infiltration in the liver which was of moderate degree at 25 mgm. per kgm. and more marked at higher doses. At 10 mgm. per kgm. no abnormal morphology could be found.

*Local tissue damage.* A one per cent solution of I-WBR-86 instilled into the eyes of rabbits produced definite irritation and local anesthesia. This solution caused no grossly observable damage when injected subcutaneously in mice. In guinea pigs it produced slight induration and petechiae, while in rats it produced moderate induration and ecchymosis and only very slight necrosis. The local tissue reactions to Pyribenzamine and Benadryl were similar but those to Benadryl were more severe in all three species.

*Antisialagogue activity.* A comparison was made of the antagonistic effects of I-WBR-86 at 15 mgm. per kgm. and atropine sulfate at 0.3 mgm. per kgm. on pilocarpine-induced salivation in a group of eight rabbits. At these doses atropine sulfate reduced the salivary output per hour per rabbit from an average control level of 28.6 cc. to 0.2 cc. whereas I-WBR-86 only reduced it to 19.1 cc. Hence I-WBR-86 appears to possess much less than one-fiftieth the antisialagogue activity of atropine sulfate.

#### SUMMARY

1. Beta-pyrrolidineethyl-phenothiazine monohydrochloride (Pyrrolazote) was compared with Pyribenzamine and demonstrated to be a potent antagonist to many of the pharmacologic responses of histamine. It was found to be effective for a longer period than Pyribenzamine as indicated by tests against histaminic spasms *in vitro* and protection against an aerosol of histamine *in vivo*.
2. Pyrrolazote was shown to possess antianaphylactic properties similar to those of Pyribenzamine.
3. Pyrrolazote neither enhanced nor antagonized the pressor effects of epinephrine in contradistinction to the enhancement produced by Pyribenzamine.
4. Acute toxicity studies in three species showed Pyrrolazote to be from one-

half to one-twentieth as toxic as Pyribenzamine depending upon the route of administration and species used.

5. Chronic toxicity studies in rats showed that a dose of 10 mgm. per kgm. orally five days each week for ten weeks was innocuous. Histopathology limited to degenerative fatty infiltration of the liver occurred at doses of 25 mgm. per kgm. and greater.

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# A PRELIMINARY PHARMACOLOGICAL INVESTIGATION OF THE TANNIN OBTAINED FROM PINUS CARIBAEA, MORELET

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Because of the success of the tannic acid method of treatment of burns, it seemed worthwhile to investigate a substance which might eliminate or at least decrease some of the objectionable properties of tannic acid, but at the same time maintain or even increase its favorable properties.

According to statistics compiled from 1,369 cases by Harkins (1), tannic acid treatment reduced mortality from 26.7 per cent to 10.5 per cent. Bettman (2), in a consecutive series of 778 cases, reported a mortality of only 3.1 per cent with combined tannic acid and silver nitrate treatment.

The disadvantages of tannic acid are: masking of underlying infection by the eschar; cracking of stiff eschar and curling of its edges; black appearance of the tanned area; delay of healing; possibility of liver damage (3). The major one of these is the possible production of liver necrosis produced by the absorption of tannic acid into the general circulation.

The pharmacological investigation of the tannin obtained from *Pinus Caribaea*, Morelet, was suggested after a consideration of the properties of this natural tannin and its ability to react with proteins in a manner similar to tannic acid (4). This experimental work gives a comparison of the relative toxicities of the phlobatannin of *Pinus Caribaea* and tannic acid, and a comparison of the effects of these two tanning agents in the local treatment of burns.

A preliminary phytochemical investigation of this phlobatannin has been carried out by two of the authors (4).

The phlobatannin used in this work was crude phlobatannin obtained by defatting the inner bark of the slash pine tree, *Pinus Caribaea*, Morelet, extracting with 95 per cent alcohol, and then removing the alcohol by vacuum distillation.

All experimental work with phlobatannin was carried out in parallel with tannic acid.

**DETERMINATION OF LD<sub>50</sub> INTRAPERITONEALLY IN MICE.** Test animals were young male and female mice varying from 18 to 22 grams in weight and averaging 19.1 grams. A total of 325 mice were used, 170 on phlobatannin, 125 on tannic acid, and 30 on saline. The work was accomplished during the late winter and early spring months and the mice were kept at room temperature, about 25°C.

Studies were conducted in groups of 5, 10, or 15 mice in each treated group and, therefore, the data in the tables represent a summation of the results obtained from several experiments. All deaths occurring within one week of injection are included. Injections were made intraperitoneally.

Phlobatannin was administered as 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2.0 per cent solutions in

<sup>1</sup> This work was presented as part of a thesis to the Graduate School at the University of Minnesota in partial fulfillment of the degree of Doctor of Philosophy.

water, tannic acid as 0.1, 0.2, 0.25, 0.3, and 0.4 per cent solutions in water. Controls were injected with 0.9 per cent solutions of sodium chloride in water.

Calculations of the LD<sub>50</sub> were made according to the method of Litchfield and Fertig (5) from dosage effect curves plotted from probits of the per cent mortality and logarithms of the doses taking into consideration the standard error of the logarithms of the LD<sub>50</sub>.

The data obtained on the acute toxicity of phlobatannin are presented in table 1, and of tannic acid in table 2. No deaths occurred in the group of 30 control mice. The LD<sub>50</sub> of phlobatannin was calculated to be  $319.9 \pm 13.2$  mgm. per kgm. The LD<sub>50</sub> of tannic acid was calculated to be  $54.32 \pm 2.25$  mgm. per

TABLE 1  
*Data for determination of LD<sub>50</sub> of phlobatannin*

DOSE mgm./kgm.	NO. OF MICE	NO. DIED	% MORTALITY	AV. TIME OF DEATH hr.
40	5	1	20.0	151
80	15	1	6.6	68
120	30	3	10.0	65
160	30	5	16.6	21
200	30	6	20.0	45
300	30	10	33.3	60
400	30	22	73.3	50

TABLE 2  
*Data for determination of LD<sub>50</sub> of tannic acid*

DOSE mgm./kgm.	NO. OF MICE	NO. DIED	% MORTALITY	AV. TIME OF DEATH hr.
20	5	0	0.0	—
40	30	2	6.6	48
50	30	10	33.3	43
60	30	19	63.3	37
80	30	29	96.6	29

kgm. The survivors were kept for 28 days. If the few additional deaths occurring in the 8 to 28 day period were included, the LD<sub>50</sub> for phlobatannin would be  $263 \pm 20.5$  mgm. per kgm., and for tannic acid  $54.96 \pm 2.10$  mgm. per kgm.

Autopsies were performed on most of the mice dying in the 28 day period. Both the phlobatannin and tannic acid mice frequently showed congested lungs. In addition several of the tannic acid mice showed hemorrhage in the pleural cavity. Fifty per cent of the phlobatannin mice showed moderate congestion of the liver, while 75 per cent of the tannic acid mice showed moderate to marked congestion of the liver. Hemorrhage in the intestines occurred in 20 per cent of the tannic acid mice as compared to 5 per cent for the phlobatannin mice. All surviving mice were sacrificed at the end of twenty-eight days and autopsied. The only gross pathologic change noted was the formation of adhesions in the

peritoneal cavity which were more frequent among the mice injected with phlobatannin than with tannic acid.

Microscopic examinations of the liver were kindly made by Drs. E. T. Bell and J. S. McCartney of the Department of Pathology. The tannic acid mice in general showed rather extensive central necrosis and severe injury to the liver. The phlobatannin mice, on the other hand, showed a peculiar diffuse hepatitis consisting of uniform infiltration with polymorphonuclear leucocytes and miliary infarcts. Some evidence was obtained suggesting that this condition was reversible.

**LOCAL THERAPY OF EXPERIMENTAL BURNS.** For the local treatment of the experimental burns, 5 and 10 per cent aqueous solutions of phlobatannin and tannic acid were used. Controls were treated with an 0.9 per cent solution of sodium chloride in water. The experimental animals were young white male rats averaging 304 grams in weight. The rats were anesthetized with 60 mgm. per kgm. of sodium pentobarbital intraperitoneally, then clipped as closely as possible on the back with scissors, washed with soap and water, shaved and dried. Burns were produced on the prepared areas by the general method described by Barnes and Rossiter (6), which consisted of holding a beaker containing boiling water firmly against the prepared area for 30 seconds as timed by a stop watch. Inasmuch as a rat does not blister when burned, the area was then immediately denuded with scissors leaving a very deep wound equivalent to a third degree burn.

The experimental work was carried out in three separate groups. Group I consisted of 8 rats which were burned by using a 50 cc. beaker, and were treated with the appropriate agent applied by cotton pledges. Groups II and III (15 and 17 rats respectively) were burned by using a 10 cc. beaker, with treatment applied by spraying from an atomizer. All controls were treated by the cotton pledge method. A total of 40 rats was used; they were kept in individual cages. Local therapy was begun 30 minutes after the burn was produced. A second application was made 20 minutes after the first, following which the burns were treated every 10 minutes until a total of seven applications was completed.

Data on Group I rats are presented in table 3. These rats received the largest burns of the three groups. All burned areas showed progress towards tanning within the first twenty-four hours, but forty-eight hours were required to cover the burn completely with an eschar by phlobatannin or tannic acid action. No controls were run in this group. The column labeled "Duration of Eschar" refers to the length of time that the original eschar remained completely intact. The "Healing Time" refers to the total days required for all traces of an eschar to be gone and the area completely healed. "Size of the Scar" refers to the maximum points of measurement of the final scar tissue after complete healing.

The appearances of the tanned areas were markedly different in the two treated groups. Tannic acid caused a typical black, rough, stiff, and tough eschar. Phlobatannin, on the other hand, produced an eschar that was more of a flesh color, about the same thickness as that of tannic acid but not quite as stiff or as rough. After loss of the original eschars, the wounds remained open about twice as long with tannic acid as with phlobatannin.

Data on the rats in Group II are presented in table 4 and on rats in Group III in table 5. The procedures were the same in both groups except that most of the animals in Group III were retreated by spraying every 10 minutes for a total of five times whenever the wound became open. In addition, Group III con-

TABLE 3  
Group I—White rats burned by contact of 50 cc. beaker

% DRUG	RAT NUMBER	COMPLETE ESCHAR	DURATION OF ESCHAR	HEALING TIME	SIZE OF SCAR	
					Long	Wide
5% Phlobatannin	1	hr.	days	days	cm.	0.3
	2	48	17	34		
5% Tannic acid	3	48	6	34	4.9	1.0
	4	48	6	47	5.1	0.9
10% Phlobatannin	5	48	7	39	4.8	0.5
	6	48	14	36	4.6	0.4
10% Tannic acid	7	48	6	36	5.0	0.6
	8	48	6	38	4.8	0.5

TABLE 4  
Group II—White rats burned by contact of 10 cc. beaker

% DRUG	RAT NUMBER	COMPLETE ESCHAR	DURATION OF ESCHAR	HEALING TIME	SIZE OF SCAR	
					Long	Wide
5% Phlobatannin	1	hr.	days	days	cm.	0.5
	2	24	9	31		
	3	48	13	31		
5% Tannic acid	4	24	11	27	0.8	0.4
	5	24	9	31		
	6	48	13	31		
10% Phlobatannin	7	24	10	31	0.7	0.5
	8	48	9	31		
	9*	24	10	died 21		
10% Tannic acid	10	24	9	29	0.5	0.2
	11	48	7	29		
	12	24	9	29		
NaCl Controls	13*	24	9	died 21	0.7	0.3
	14	48	9	27		
	15	48	9	29		

\* Indicates rats that were accidental deaths.

tained 2 rats that were treated with a 5 per cent and a 10 per cent solution of the phlobatannin that had been stored in a refrigerator for six years. Neither retreatment nor the age of the phlobatannin had any significant effect on the

healing of the burns. Rat No. 8 became infected and was sacrificed on the thirty-first day. An autopsy showed all organs to be normal in appearance, even though the burn was retreated a number of times.

From the results obtained in the treatment of burns as given in the above three tables, it may be noted that eschars were usually formed in twenty-four to forty-eight hours in all groups but tended to remain completely intact longer on the

TABLE 5  
*Group III—White rats burned by contact of 10 cc. beaker*

% DRUG	RAT NUMBER	COMPLETE ESCHAR	DURATION OF ESCHAR	HEALING TIME	SIZE OF SCAR		DAY RETREATED
					Long	Wide	
5% Phlobatannin	1	48	13	36	2.0	1.4	
	2*	48	8	31	1.5	0.5	8
	3*	48	8	36	1.8	1.2	8
5% Tannic acid	4	72	13	32	2.1	0.8	
	5*	48	7	31	2.5	0.5	13
	6*	48	6	31	1.5	0.7	6, 7
10% Phlobatannin	7	48	14	31	1.5	0.7	
	8*	48	7	Infected killed			7, 12, 18, 20, 25
	9*	48	7	31	1.8	0.6	7, 12
10% Tannic acid	10	48	7	36	2.1	0.6	
	11*	48	6	36	2.2	0.8	6
	12*	48	6	36	1.6	1.3	7, 12
NaCl Control	13*	48	6	31	1.3	0.7	13
	14*	48	11	31	1.5	1.2	13
	15	72	8	31	1.8	0.8	
5% Old Phlobatannin	16	48	7	36	1.8	0.8	
10% Old Phlobatannin	17	48	13	31	1.9	0.7	

\* Indicates burns retreated as soon as they became open.

phlobatannin rats than on the tannic acid or control rats. However, little difference was evident in the total time for complete healing in the three groups. The smallest amount of final scar tissue was produced by 10 per cent phlobatannin.

#### SUMMARY

Tannic acid, injected intraperitoneally into mice, caused necrosis of the liver whereas phlobatannin produced a different type of liver injury, namely, a diffuse hepatitis.

The LD<sub>50</sub> of tannic acid intraperitoneally in mice was calculated to be 54.32 ± 2.25 mgm./kgm. whereas the LD<sub>50</sub> of the phlobatannin of *Pinus Caribaea*, Morelet, was 319.9 ± 13.2 mgm./kgm. Thus phlobatannin has approximately one-sixth of the acute toxicity of tannic acid.

In the local treatment of burns, solutions of phlobatannin and of tannic acid were about equal in respect to time required for the formation of a complete eschar over the burned area. However, the eschars formed with phlobatannin remained completely intact slightly longer, were less stiff and brittle and were lighter in color than those produced by tannic acid.

This experimental work indicates that *Pinus Caribaea* phlobatannin possesses the beneficial properties of tannic acid in the local therapy of burns, but is decidedly less toxic.

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# THE RELATIONSHIP OF CHOLINESTERASE INHIBITING ACTIVITY TO THE TOXICITY OF SOME ORGANIC PHOSPHORUS COMPOUNDS<sup>1</sup>

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The recent experimental clinical use of diisopropyl fluorophosphate (DFP) in myasthenia gravis (1, 2), glaucoma (3), and paralytic ileus (4), and the proposed use of hexaethyl tetraphosphate (HETP) and tetraethyl pyrophosphate (TEPP) as insecticides and rodenticides (5, 6) has focused attention on the anti-enzymatic activity as well as the toxicity of organic phosphorus compounds. For example, DFP (7, 8), HETP (5, 9, 10), and TEPP (6, 10) are all known to be potent inhibitors of cholinesterase and to possess a high degree of toxicity. That the pharmacological effects resulting from the administration of DFP to animals and man are associated with inhibition of tissue cholinesterases has been demonstrated by the studies of Modell et. al. (11), Koelle and Gilman (12), Mazur and Bodansky (7), Comroe et. al. (1, 13), Harvey et. al. (2, 4, 14, 15), and Nachmansohn (16), but it has not been fully established that the toxicity of DFP and other anti-cholinesterases is entirely dependent on their cholinesterase inhibiting properties. Should other factors be in part or wholly responsible for the toxicity of these compounds, then additional organic phosphorus compounds might be found which would be potent cholinesterase inhibitors but relatively less toxic than those so far investigated, and, therefore, of potential clinical use. To elucidate further the relationship between anti-cholinesterase activity and toxicity, the degree of *in vitro* inhibition of rat brain cholinesterase caused by 43 organic phosphorus compounds was compared with their intraperitoneal toxicity to mice. Thirty-eight of these inhibited cholinesterase weakly or not at all and were relatively non-toxic. These will not be considered further in this report.

**MATERIALS AND METHODS.** 1. *Anti-Cholinesterase Activity.* Cholinesterase activity was determined by a modification of the method of Ammon (17). The hydrolysis of acetylcholine was followed manometrically by means of the liberation of carbon dioxide from a Krebs-Ringer-bicarbonate buffer (18) in Warburg vessels. The concentration of acetylcholine in the final volume of 4.0 cc. was 0.015 M. The source of enzyme was 1.0 cc. of the centrifuged supernatant from a rat brain-buffer homogenate prepared in the Waring blender. The amount of the inhibiting agent calculated to give the desired final concentration was introduced in 0.1 cc. of propylene glycol, and this amount of propylene glycol was also included in the control vessels. After gassing the vessels with 95 per cent nitrogen—5 per cent carbon dioxide for ten minutes and equilibrating at 37°C. for 10 minutes, the acetylcholine was tipped into the main vessel from the side arm. Readings were taken

<sup>1</sup> A preliminary report of this work appeared in Fed. Proc., 7: 231, 1948.

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at 10, 40 and 70 minutes after tipping. Percentage of inhibition was calculated by comparison with controls containing the same enzyme preparation without the inhibitors. A correction was made for the nonenzymatic hydrolysis of acetylcholine and, in one case (Compound No. 2, table 1), it was necessary to make an allowance for a gas which was given off by the inhibitor during the determination. All of the compounds were tested in a series of dilutions from which the concentration causing 50 per cent inhibition was determined graphically.

**2. Toxicity.** The toxicity of all compounds was tested by intraperitoneal injection in C.F. albino mice weighing between 17 and 27 grams. Median lethal doses ( $LD_{50}$ 's) were calculated by the method of Bliss (19) using mortality data obtained by the injection of groups of six mice at each of six dose levels. The period of observation, following a single injection, was seven days. Toxicity studies and determinations of cholinesterase inhibiting

TABLE 1  
*Anti-cholinesterase activity and toxicity*

NO.	NAME	FORMULA	M. CONC. FOR 50% INHIBITION CHOLINESTERASE	$LD_{50} \pm 1$ S.E.	$LD_{50}$
1.	Tetraethyl pyrophosphate	$(C_2H_5O)_2POOP(OC_2H_5)_2$	$6.3 \times 10^{-9}$	$1.16 \pm .11$	4.00
2.	—	$[(C_2H_5O)_2POO]_2 POCH_3$	$1.0 \times 10^{-8}$	$3.49 \pm .17$	9.48
3.	Diisopropyl fluorophosphate	$FPO[OCH(CH_3)_2]$	$4.3 \times 10^{-7}$	$8.96 \pm 1.04$	48.6
4.	Diisopropyl chlorophosphate	$ClPO[OCH(CH_3)_2]$	$2.0 \times 10^{-6}$	$29.2 \pm 1.3$	146
5.	p-Chlorophenyl diethoxy phosphine oxide	$p-ClC_6H_4PO(OC_2H_5)_2$	$1.6 \times 10^{-5}$	$138 \pm 25$	562

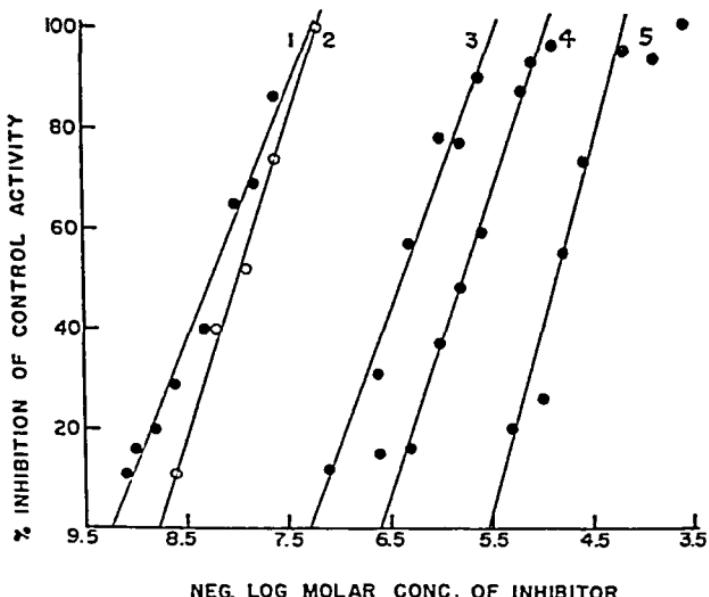
activity were carried on simultaneously when compounds which were thought to be unstable were being investigated.

**RESULTS.** The five compounds that proved to be potent inhibitors of cholinesterase were tetraethyl pyrophosphate,  $[(C_2H_5O)_2POO]_2POCH_3$ , diisopropyl fluorophosphate, diisopropyl chlorophosphate, and p-chlorophenyl diethoxy phosphine oxide.<sup>4</sup> The percentage inhibition of cholinesterase obtained with varying concentrations of each of these compounds is presented graphically in fig. 1. Since the midportions of the resulting curves appeared to be linear, the straight lines that would best fit the experimental points were calculated by the method of least squares. It is interesting to note that the slopes of the lines are nearly identical. This may indicate a similar mechanism of reaction between cholinesterase and each of these inhibitors. All seven compounds caused 50 per cent inhibition of cholinesterase activity in concentrations of  $1.6 \times 10^{-5}$  molar or less, as shown in table 1.

<sup>4</sup>This compound was the only one of the five potent inhibitors that regularly resulted in delayed mortality, deaths occurring anytime from a few minutes to five days after injection. The pathologic effects of this compound are described elsewhere (20).

The results of the toxicity determinations are also presented in table 1. When the negative logs of the molar concentrations of these compounds causing 50 per cent inhibition of cholinesterase were plotted against the logs of the LD<sub>50</sub>'s in micromols, a straight line was obtained (fig. 2). The coefficient of correlation of these two variables is 0.99 with one chance in 1000 that it is less than 0.87.

**DISCUSSION.** It was not expected that the *in vitro* cholinesterase inhibiting activity as determined in these studies would bear a precise relationship to toxicity. *In vivo*, the rate of absorption of the compounds from the site of injection, the rate of hydrolysis, of enzymatic detoxification, and of excretion, and the solu-



NEG. LOG MOLAR CONC. OF INHIBITOR

Fig. 1. Cholinesterase inhibition vs. concentration of inhibitor. 1. Tetraethyl pyrophosphate, 2.  $[(C_2H_5O)_2POCH_2]_2POCH_2$ , 3. Diisopropyl fluorophosphate, 4. Diisopropyl chlorophosphate, 5. p-Chlorophenyl diethoxy phosphine oxide.

bility in the lipids of the nervous system might all affect the degree of cholinesterase inhibition. Du Bois et. al. (21) found that the ratio of *in vitro* to *in vivo* cholinesterase inhibiting activity varied in a group of drugs somewhat more heterogenous than the one studied here.

It is striking, therefore, that a correlation can be made between the degree of cholinesterase inhibition and the toxicity of the five potent anti-cholinesterases investigated in this study. This was shown by the straight line relationship between the logs of the LD<sub>50</sub>'s and the logs of the concentrations causing 50 per cent inhibition.

Dixon and Needham (22) tested the effects of DFP and other fluorophosphates on a number of enzyme systems and found that only cholinesterase was inhibited. They stated that these drugs are the most powerful and specific enzyme inhibitors known.

Modell et. al. (11), considering the cause of death resulting from DFP poisoning, concluded, on the basis of an investigation of the general systemic effects of

at 10, 40 and 70 minutes after tipping. Percentage of inhibition was calculated by comparison with controls containing the same enzyme preparation without the inhibitors. A correction was made for the nonenzymatic hydrolysis of acetylcholine and, in one case (Compound No. 2, table 1), it was necessary to make an allowance for a gas which was given off by the inhibitor during the determination. All of the compounds were tested in a series of dilutions from which the concentration causing 50 per cent inhibition was determined graphically.

*2. Toxicity.* The toxicity of all compounds was tested by intraperitoneal injection in C.F. albino mice weighing between 17 and 27 grams. Median lethal doses ( $LD_{50}$ 's) were calculated by the method of Bliss (19) using mortality data obtained by the injection of groups of six mice at each of six dose levels. The period of observation, following a single injection, was seven days. Toxicity studies and determinations of cholinesterase inhibiting

TABLE 1  
*Anti-cholinesterase activity and toxicity*

NO.	NAME	FORMULA	M. CONC. FOR 50% INHIBI- TION CHOLIN- ESTERASE	$LD_{50} \pm 1 S.E.$	$LD_{50}$
1.	Tetraethyl pyro- phosphate	$(C_2H_5O)_2POOP(OC_2H_5)_2$	$6.3 \times 10^{-9}$	$1.16 \pm .11$	4.00
2.	—	$[(C_2H_5O)_2POPO]_2 POCH_3$	$1.0 \times 10^{-8}$	$3.49 \pm .17$	9.48
3.	Diisopropyl fluoro- phosphate	FPO[OCH(CH <sub>3</sub> ) <sub>2</sub> ] <sub>2</sub>	$4.3 \times 10^{-7}$	$8.96 \pm 1.04$	48.6
4.	Diisopropyl chloro- phosphate	ClPO[OCH(CH <sub>3</sub> ) <sub>2</sub> ] <sub>2</sub>	$2.0 \times 10^{-6}$	$29.2 \pm 1.3$	146
5.	p-Chlorophenyl di- ethoxy phosphine oxide	p-ClC <sub>6</sub> H <sub>4</sub> PO(OC <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	$1.6 \times 10^{-5}$	$138 \pm 25$	562

activity were carried on simultaneously when compounds which were thought to be unstable were being investigated.

**RESULTS.** The five compounds that proved to be potent inhibitors of cholinesterase were tetraethyl pyrophosphate,  $[(C_2H_5O)_2 OPO]_2 POCH_3$ , diisopropyl fluorophosphate, diisopropyl chlorophosphate, and p-chlorophenyl diethoxy phosphine oxide.<sup>4</sup> The percentage inhibition of cholinesterase obtained with varying concentrations of each of these compounds is presented graphically in fig. 1. Since the midportions of the resulting curves appeared to be linear, the straight lines that would best fit the experimental points were calculated by the method of least squares. It is interesting to note that the slopes of the lines are nearly identical. This may indicate a similar mechanism of reaction between cholinesterase and each of these inhibitors. All seven compounds caused 50 per cent inhibition of cholinesterase activity in concentrations of  $1.6 \times 10^{-5}$  molar or less, as shown in table 1.

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**DISCUSSION.** It was not expected that the *in vitro* cholinesterase inhibiting activity as determined in these studies would bear a precise relationship to toxicity. *In vivo*, the rate of absorption of the compounds from the site of injection, the rate of hydrolysis, of enzymatic detoxification, and of excretion, and the solu-

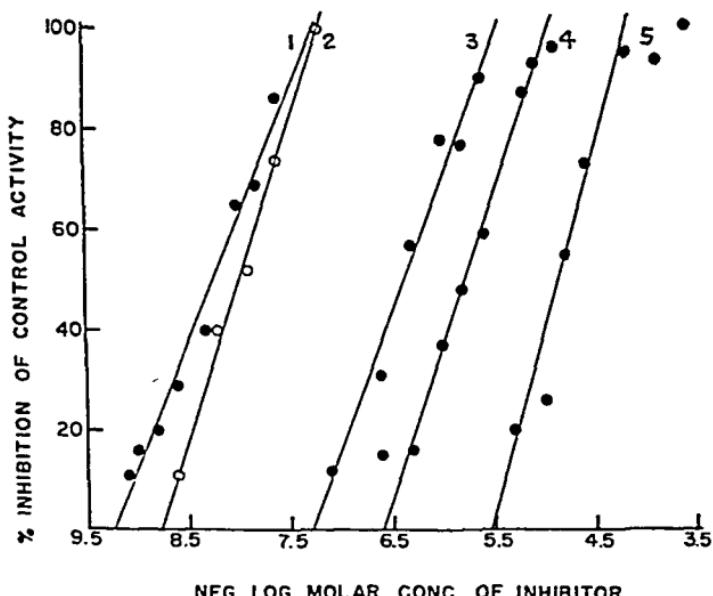


FIG. 1. Cholinesterase inhibition vs. concentration of inhibitor. 1. Tetraethyl pyrophosphate, 2.  $[(C_2H_5O)_2PO]_2 POCH_3$ , 3. Diisopropyl fluorophosphate, 4. Diisopropyl chlorophosphate, 5. p-Chlorophenyl diethoxy phosphine oxide.

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Modell et. al. (11), considering the cause of death resulting from DFP poisoning, concluded, on the basis of an investigation of the general systemic effects of

this drug in cats, that most of the important systems of the body are affected by DFP, and that the major action which leads to these changes is probably the inhibition of cholinesterase. They noted, however, that death after massive doses of DFP in animals protected with atropine seems to involve a combination of central and peripheral paralysis of a nature similar to that caused by nicotine.

The investigations of Koelle and Gilman (12), Comroe et al. (1, 13), Harvey et al. (2, 4, 14, 15), and others indicate that the pharmacological and clinical

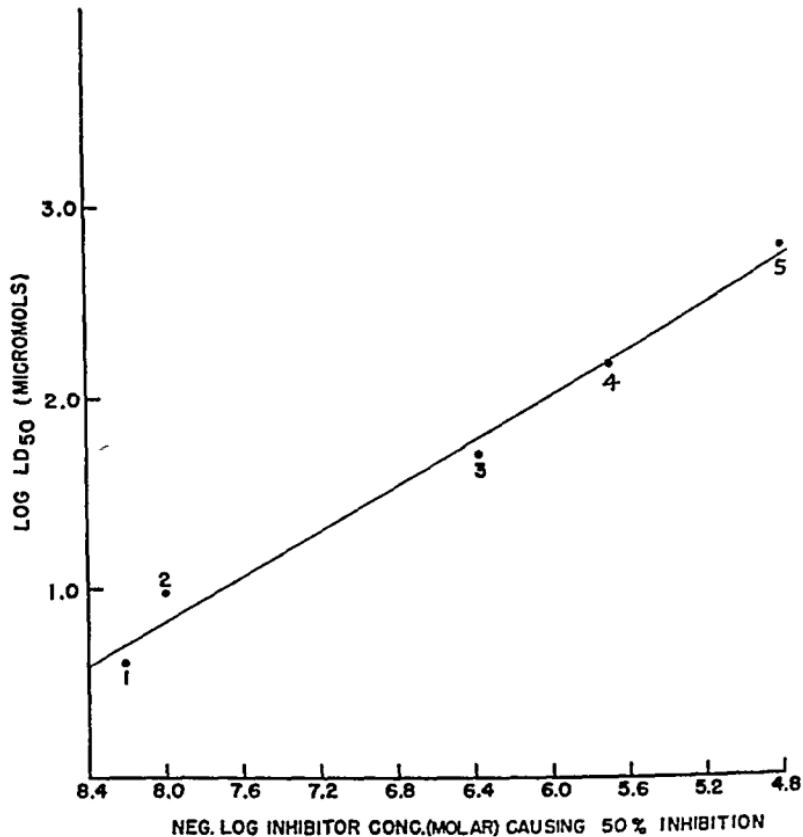


FIG. 2. Relationship of anticholinesterase activity to toxicity. 1. Tetraethyl pyrophosphate, 2.  $[(C_2H_5O)_2POCH_2]_2$ , 3. Diisopropyl fluorophosphate, 4. Diisopropyl chlorophosphate, 5. p-Chlorophenyl diethoxy phosphine oxide.

effects of DFP are associated with low red blood cell and brain cholinesterase levels. Mazur and Bodansky (7) noted, following the injection of DFP into rabbits and monkeys, that the brain cholinesterase level was zero in animals that died, but several of the survivors also showed zero or negligible brain cholinesterase levels. Nachmansohn and Feld (16), on the contrary, recently reported that, in a group of rabbits injected with a particular dose of DFP, all those that died showed zero or negligible brain cholinesterase levels while, in survivors, cholinesterase was always present in from 10 to 50 per cent of the normal amounts in

the brain. They believe that the difference between their results and those of Mazur and Bodansky is due to certain refinements in technique which they describe. Nachmansohn and Feld conclude that, in view of the high specificity of action of DFP and its powerful effect on cholinesterase in low concentrations, the coincidence of death and inactivation of cholinesterase suggest that the toxicity of the compound is due to its action on the enzyme.

Chadwick and Hill (23) have recently demonstrated that DFP and HETP are potent inhibitors of the cholinesterase activity of roach nerve cord *in vitro* and *in vivo*. They found that the percentage of mortality after injection of DFP was closely paralleled by the degree of enzyme inhibition which resulted from the same dosage. They concluded that, although the possibility of other toxic mechanisms for these agents is not excluded, their toxicity is largely a function of their anticholinesterase activity.

Miller and Ginsberg (24), having demonstrated that survivors and non-survivors of LD<sub>50</sub> doses of a prostigmine analogue and DFP showed no significant difference in the free acetylcholine levels of either brain or sub-maxillary gland, concluded that the amount of acetylcholine liberated at the site of action of a cholinergic drug is not the primary factor responsible for the physiologic activity of such a drug. This conclusion might be questioned since there seems to be no reason to expect a significant difference in the free acetylcholine found in the tissues of survivors and non-survivors of an LD<sub>50</sub> dose. It is well known that individuals within a group may vary in their sensitivity to acetylcholine.

The fact that, in the present study, the logs of the LD<sub>50</sub>'s of a group of anticholinesterases of a wide range of toxicity were proportional to the logs of the concentrations necessary to produce 50 per cent inhibition of a brain cholinesterase preparation, may be interpreted as additional evidence that the toxicity of these compounds is primarily due to the inhibition of cholinesterase.

#### SUMMARY

1. The degree of *in vitro* inhibition of rat brain cholinesterase caused by five organic phosphorus compounds is compared with their intraperitoneal toxicity to mice. These compounds are: (1) tetraethyl pyrophosphate, (2) [(C<sub>2</sub>H<sub>5</sub>O)<sub>2</sub>OPO]<sub>2</sub>POCH<sub>3</sub>, (3) diisopropyl fluorophosphate, (4) diisopropyl chlorophosphate, (5) p-chlorophenyl diethoxy phosphine oxide. The concentrations in which these compounds cause 50 per cent inhibition of cholinesterase activity and their LD<sub>50</sub>'s in micromols and mgm. per kgm. are presented in tabular form.

2. The logs of the LD<sub>50</sub>'s of these five potent cholinesterase inhibitors are proportional to the logs of the molar concentrations causing 50 per cent inhibition of cholinesterase. The significance of this finding is discussed.

The authors wish to express their appreciation to Mr. Jack P. Saunders and Doctor Williamina A. Himwich for technical advice, to Miss Frieda F. Faiman and Mrs. Lottie K. Jandorf who did the statistical analyses, and to the Technical Command, Army Chemical Center, and the Monsanto Chemical Company, who supplied the compounds investigated in this study.

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# THE EFFECT OF ADRENERGIC BLOCKING AGENTS ON THE VASOCONSTRICTION PRODUCED BY ACUTE OXYGEN LACK<sup>1</sup>.

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Since the original discovery that ergot alkaloids would block the excitatory effects of the sympatho-adrenal system (1), many drugs have been found which possess this activity. Extensive studies have been carried out to show the antagonistic action of these drugs against epinephrine and similar sympathomimetic amines and against direct stimulation of sympathetic nerves (2-5). Comparatively few experiments have been carried out on reflexly induced sympathetic effects. We have investigated the effects of certain typical adrenergic blocking agents<sup>2</sup> against the blood pressure rise produced by temporary acute oxygen lack.

**EXPERIMENTAL PROCEDURE.** Sixteen apparently healthy, adult mongrel dogs (6.1-14 kgm.) were used; these were anesthetized with sodium barbital (330 mgm./kgm.) administered intraperitoneally 90 minutes prior to use. One-half the animals also received 1 mgm. atropine sulfate per kgm. Blood pressure was recorded by the usual mercury manometer and soot kymograph or by ink-writing mercury manometer and continuous strip kymograph. The trachea was cannulated. Test doses of 2.2 micrograms of epinephrine hydrochloride per kgm. were injected intravenously into an exposed femoral vein. This was followed, after a five minute rest period, by 0.2 mgm. of ephedrine hydrochloride per kgm. After an additional rest period, the tracheal cannula was connected for 60 seconds to a gas reservoir containing 7 per cent oxygen-93 per cent nitrogen by volume (we are grateful to Dr. J. C. Stickney for the control gas analyses and for calibration of the apparatus). Flutter valves were so arranged that the animal could only inhale from the reservoir and only exhale to the outside atmosphere. Some of the early experiments were also run with atmospheres containing 5 per cent and 9 per cent oxygen. Some animals were exposed to simple asphyxia by closing the inlet valve for 30 seconds. One mgm. of adrenergic blocking agent per kgm. was administered intravenously. After the blood pressure level stabilized (5-20 minutes), the series: epinephrine, ephedrine, and anoxic anoxia<sup>3</sup> were repeated. Additional doses of blocking agent were administered and the procedure repeated as long as the animal had a mean blood pressure above 50 mm. Hg. In the experiments with  $\alpha$ -naphthyl-

<sup>1</sup> Part of the material in this paper was presented before the meetings of the Federation of American Societies for Experimental Biology, Atlantic City, 1946. See Federation Proceedings 5: 10G, 1946.

<sup>2</sup> We are grateful to the Sandoz Chemical Works, New York, for generously supplying the ergotamine tartrate, to D. Bovet of the Institut Pasteur, Paris, for the piperidinomethyl-benzodioxan, and to G. Rieveschl, Jr., of Parke, Davis and Company, Detroit, for the  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine. Other drugs were purchased on the open market.

<sup>3</sup> The term "anoxic anoxia" is used to designate hypoxia without accompanying increase in carbon dioxide concentration.

methylethyl- $\beta$ -chloroethylamine, 2.2 micrograms histamine (base) per kgm. was substituted for the ephedrine. A portion of a typical experiment is reproduced in figure 1.

**RESULTS.** In general, the results with a given adrenergic blocking agent are uniform and individual animal variation does not seem to be a factor. Typical results are given in table 1. The effects with 5 per cent and 9 per cent oxygen are qualitatively the same, the differences being in amount of response, with the 5 per cent oxygen giving more pronounced changes, and the 9 per cent oxygen often giving only slight changes in blood pressure. The most startling result is observed with low doses of ergotamine tartrate in which reversal of the anoxic anoxia and asphyxia induced rises occurs even though the response to epinephrine and ephedrine are both pressor. With the other agents, reversal of the anoxic

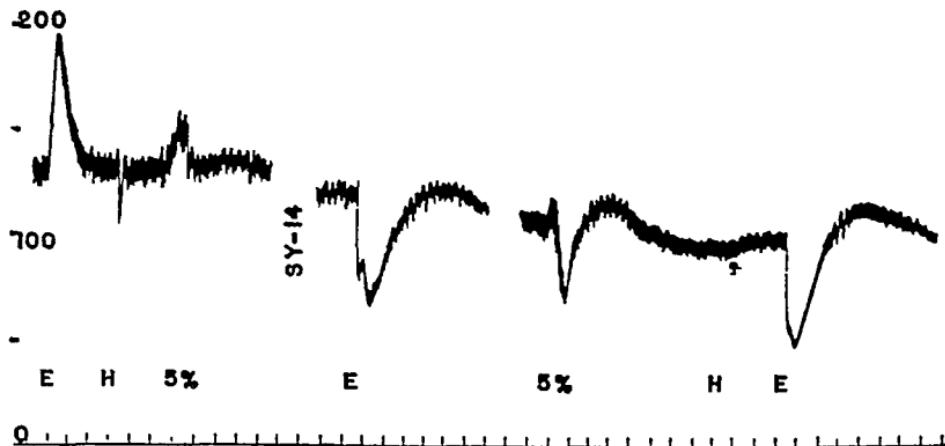


FIG. 1. Dog (Female; 10 kgm.). 330 mgm. Na barbital per kgm. 1 mgm. atropine sulfate per kgm. Pressure scale in mm. Hg from femoral artery cannula; time scale in minutes. 2.2 micrograms epinephrine hydrochloride per kgm. injected intravenously at E, 2.2 microgram histamine base per kgm. at H, and connected to 5 per cent oxygen-95 per cent nitrogen via tracheal cannula at 5 per cent. Five mgm.  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine hydrochloride injected intravenously at SY-14 and 18 minutes of record removed until blood pressure levelled off. Clot washed out during other blank space in record.

anoxia induced rise occurs at dose levels which are termed "sympatholytic" (4, 6) and block the pressor effects of ephedrine, rather than at dose levels which are termed "adrenolytic" and only reverse the action of epinephrine.

**DISCUSSION.** That anoxic anoxia (oxygen lack without carbon dioxide increase) can produce a rise in blood pressure has been known for a long time (7) and has been the subject of much investigation. It seems generally accepted that the rise is primarily due to the activity of the carotid sinus referred to the hypothalamic vasomotor centers and mediated through the sympathetic nervous system (8). Whether adrenal discharge of sympathin and epinephrine is the primary important factor has been the subject of considerable controversy (9). Regardless of the final step, if the blood pressure rise is due to either diffuse peripheral sympathetic discharge or massive discharge of epinephrine from the adrenal, the adrenergic blocking agents should prevent the rise. Our experiments

indicate that they do prevent this rise, and that after their administration they either allow or cause the blood pressure to fall when the blood oxygen level is diminished.

Bülbbring, Burn and De Elio (10) have recently demonstrated an increased outflow of epinephrine from the denervated adrenal gland perfused with oxygen deficient blood. It would be very convenient to postulate that the antagonism of the rise produced by anoxic anoxia and its subsequent conversion to a fall by adrenergic blocking agents is simply due to the release of epinephrine and a typical reversal of the action of epinephrine. The data available do not entirely substantiate such conclusions. Our results indicate that doses of ergotamine

TABLE I

	ONE MG.M./KGM.			FIVE MG.M./KGM.			TEN MG.M./KGM.		
	Reversal of 2.2 microgram/kgm. epinephrine hydrochloride	80% block of 0.2 mgm./kgm. ephedrine hydrochloride	Reversal of 7% oxygen rise	Reversal of 2.2 microgram/kgm. epinephrine hydrochloride	80% block of 0.2 mgm./kgm. ephedrine hydrochloride	Reversal of 7% oxygen rise	Reversal of 2.2 microgram/kgm. epinephrine hydrochloride	80% block of 0.2 mgm./kgm. ephedrine hydrochloride	Reversal of 7% oxygen rise
Ergotamine Tartrate	-	-	+	+	+	+	+	+	+
Yohimbine Hydrochloride	+	-	-	-	+	+	+	+	+
Piperidinomethylbenzodioxan Sulfate (933F)	+	-	-	-	+	-	-	+	+
$\alpha$ -Naphthylmethylethyl- $\beta$ -chlo- roethylamine Hydrochloride (SY-14)	+	+	+	+	+	+	+	+	+

+ indicates that the stated result occurred at this dose, - indicates that it did not occur, and blank indicates that the animals would not tolerate this dose of agent.

\* Two animals responded with reversal and two did not; all four showed reversal at 9 per cent oxygen.

which do not reverse the action of epinephrine are still able to inhibit the anoxic anoxia rise and convert it to a fall. This inhibition of rise is in agreement with the finding of Heymans and Regniers (11) that ergotamine blocks the carotid sinus reflexes and of Gellhorn (8) that denervation of the carotid sinus leads to lowered blood pressure in anoxic animals. The anomalous response of epinephrine and ephedrine may be similar to the experiments observed by Raymond-Hamet (12) in which he found that doses of ergotamine which did not reverse the effects of epinephrine did convert the usual rise in pressure following cervical sympathetic nerve stimulation to a fall.

The other autonomic modifying agents follow a more distinct pattern. Doses which are only "adrenolytic" and reverse the action of epinephrine, but do not particularly antagonize ephedrine, do not effectively antagonize the anoxic

anoxia rise, while doses which are large enough to be considered "sympatholytic" and antagonize the ephedrine rise do convert the anoxic anoxia rise to a fall. This is in agreement with the concept of Bacq and Fredericq (13) that many sympatholytic agents act peripherally, and can, in adequate dosage, block any excitatory sympathetic effect, allowing any underlying inhibitory sympathetic activity to become apparent. It is also in agreement with the observations of Nickerson and Goodman (14) with dibenzylchloroethylamine. That the fall in pressure is not histaminic in character can be demonstrated with  $\alpha$ -naphthylmethyllethyl- $\beta$ -chloroethylamine. Achenbach (15) has indicated that this agent is both antihistaminic and sympatholytic. As shown in figure 1, this drug completely antagonizes a small dose of histamine, reverses the effects of epinephrine and anoxic anoxia, but since it does not antagonize the ensuing fall from the anoxic anoxia, presumably this fall is not histaminic. Since this fall is not antagonized by atropine, it is probably not cholinergic. Consequently, it must be due either to the activity of sympathetic inhibitory effectors or act through some non-specific effects from the lack of oxygen for the peripheral vascular system.

#### SUMMARY

The rise in blood pressure following the exposure of barbitalized dogs to atmospheres containing diminished amounts of oxygen can be converted to a fall by the administration of ergotamine tartrate, yohimbine hydrochloride, piperidino-methylbenzodioxan sulfate, or  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine hydrochloride.

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# THE COMPARATIVE PHARMACOLOGY OF THE ISOMERIC HEPTYLAMINES<sup>1</sup>

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Since the fundamental study of Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. Although Barger and Dale demonstrated the activity of some of the simple aliphatic amines, little (2-4) was done with this type of agent until Rohrmann and Shonle (5) prepared a large enough group of compounds for systematic investigation to be undertaken. Swanson and Chen (6) have extensively bioassayed these agents on pithed dogs, and although one of the agents has been introduced clinically (7), very little is known of the general pharmacology of the compounds.

In this study, the various heptylamines<sup>2</sup> have been compared with each other. By limiting the problem primarily to compounds with a total of seven carbon atoms, it is possible to determine the relationship between spatial configuration and pharmacological activity, without having to consider differences in molecular weight.

**BLOOD PRESSURE EFFECTS.** 1. *Methods.* Crude preliminary experiments on barbiturized dogs indicated that repeated injections of 2-heptylamine give diminished effects as do amphetamine (3) and ephedrine (8) and that cross tachyphylaxis between the compounds under investigation can occur. In order to avoid the possible error that tachyphylaxis can introduce, the following procedures were used:

**Anesthetized dogs.** Sixty-three apparently healthy, adult mongrel dogs (4.5-19 kgm.) were used; these were anesthetized with sodium barbital (330 mgm./kgm.) administered intraperitoneally 90 minutes prior to use. The animals were either vagotomized or received 1 mgm. atropine sulfate per kgm. intravenously. Blood pressure was recorded with the usual mercury manometer and soot kymograph. The animals were standardized with graded doses of epinephrine (1-8 micrograms/kgm.) and the test dose of the agent, as a 1 per cent solution of the sulfate, was injected into the femoral vein. Five dogs were used for each compound. Part of a typical experiment is reproduced in fig. 1.

**Unanesthetized dogs.** Seven adult dogs (13-17 kgm.) were used; these received 1 mgm. scopolamine hydrobromide and 1 mgm. morphine sulfate per kgm. intraperitoneally 45 minutes prior to use. One cc. of 1 per cent procaine hydrochloride was administered in-

<sup>1</sup> This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association. Part of the material in this paper was summarized in Fed. Proc., 5: 264, 1946.

<sup>2</sup> I am grateful to Dr. K. K. Chen and Mr. H. A. Shonle, Eli Lilly and Company, Indianapolis, for generously supplying these compounds. The only missing compound in the series of methyl substituted 2-heptylamines, 2-methyl-2-heptylamine, was synthesized in this laboratory by a series of reactions analogous to those used by Zenitz, Macks, and Moore, J. Am. Chem. Soc., 70: 955, 1948, for the preparation of 1-phenyl-2-methyl-2-propylamine. I am grateful to D. A. Herring and F. K. Hampton for technical assistance.

tradernally over the femoral artery. A  $\frac{1}{2}$  inch, 20 gage hypodermic needle fitted directly to a Lambert-Wood (9) strain-gage manometer connected to a GE 8CE1DJ11 recording microammeter was used to measure the femoral arterial blood pressure. Each animal received 1, 2, 4, 5, or 6 micrograms epinephrine hydrochloride per kgm. and 10 minutes later 1 mgm. of one of the amines per kgm. The procedure was repeated every fourth day until all the agents had been administered in rotation at least once. Preliminary investigations with two dogs indicated that 2-heptylamine could be given in this manner as often as once a day with variations in response of only  $\pm 6$  per cent.

**2. Results.** The results of the epinephrine-agent equivalence are summarized in table 1. The quantitative difference between the results for barbitalized dogs (6) led to the adoption of the cross-over assay of these agents in unanesthetized

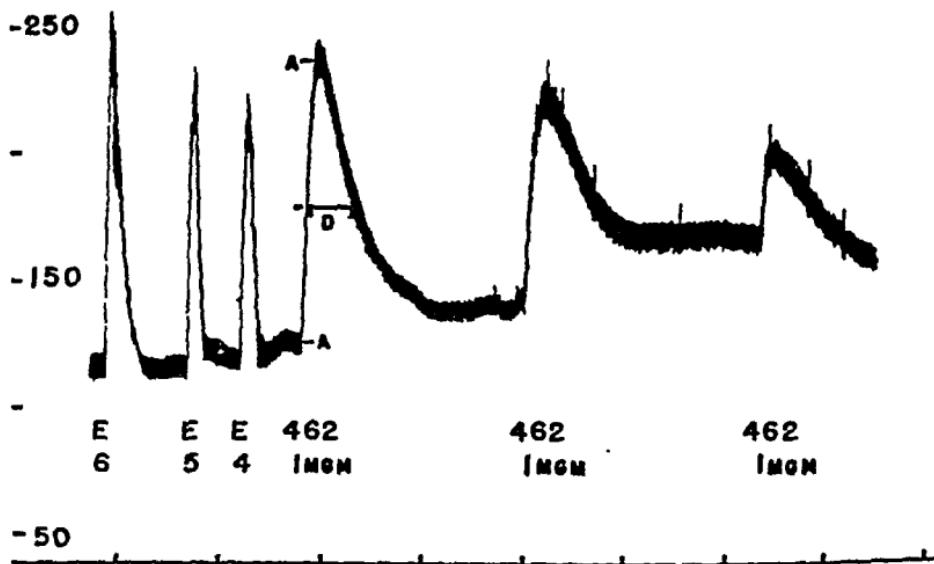


FIG. 1. Dog (Female; 10 kgm.). 330 mgm. Na Barbital per kgm.; 1 mgm. atropine sulfate per kgm. Blood pressure in mm. Hg, ordinate; time, 10 minute intervals, abscissa. E = epinephrine (base), with dose in micrograms/kgm.; 462 = 4-methyl-2-hexylamine sulfate, 1 mgm./kgm.

dogs. Since the results from these animals also differed, the records of the pithed dogs were re-measured.<sup>3</sup> Immediately the causes of the discrepancy became apparent. In the pithed dog, 2 micrograms epinephrine per kgm. gives the same rise in blood pressure ( $81.0 \pm 23$  mm. Hg) as 5 micrograms epinephrine per kgm. does in the barbitalized dogs ( $77.7 \pm 14$  mm. Hg). It only requires 5 micrograms epinephrine per kgm. in the pithed dog to give the same rise as 12 micrograms/kgm. in the barbitalized dog ( $123.7 \pm 28$  mm. Hg). However, the actual rise in mm. Hg following a 1 mgm. dose of a potent heptylamine, such as 4-methyl-2-hexylamine, is very similar in all three preparations. The procedures differ in epinephrine response more than amine pressor response. Also, the relative order

\* I am grateful to Mr. E. E. Swanson and Dr. K. K. Chen, Eli Lilly and Company, Indianapolis, for generously loaning 143 kymograph records for analysis.

TABLE 1

CHEMICAL NAME	FORMULA	PRESSOR ACTIVITY			DURATION		RABBIT JEJUNUM
		l-Epinephrine equivalent to one mgm. amine sulfate			Time for pressor rise to decrease to 50% above normal		
		Pithed Dogs*	Barbiturized Dogs	Unanesthetized Dogs	Barbiturized Dogs	Unanesthetized Dogs	Change in tone produced by 40 mgm./liter
3-Heptylamine	 <chem>CN(CCC(C)C)C</chem>	μgm. 0.15	μgm. 0.5±	μgm. 0.6±	min. 4	min. 2	+100
2-Methyl-2-hexylamine	 <chem>CN(C(C)C)CC</chem>	—	0	0.4±	—	2	+10
3-Methyl-2-hexylamine	 <chem>CN(C(C)CC)C</chem>	0.86	1.1	2.3	3	2.5	+70
4-Methyl-2-hexylamine	 <chem>CN(C(C)CCC)C</chem>	3.5	4.4	4.9	10	6	+40
5-Methyl-2-hexylamine	 <chem>CN(C(C)CCCC)C</chem>	1.56	2.9	3.5	10	7	+60
2-Heptylamine . . .	 <chem>CN(CCC(C)C)C</chem>	3.10	4.1	4.5	7	4.5	+100
4-Heptylamine . . .	 <chem>CN(C(C)CCC)C</chem>	0.05	0.4	—	0.5	—	0

\* From Swanson and Chen (6).

TABLE I—Continued

CHEMICAL NAME	FORMULA	PRESSOR ACTIVITY			DURATION		RABBIT JEJUNUM Change in tone produced by 40 mgm./liter
		1-Epinephrine equivalent to one mgm. amine sulfate			Barbiturated Dogs	Unanesthetized Dogs	
		Pithed Dogs*	Barbiturated Dogs	Unanesthetized Dogs			
1-Heptylamine.....	<p style="text-align: center;"> <math>\begin{array}{c} \text{C} \\   \\ \text{C}-\text{C} \\   \\ \text{C}-\text{C} \\   \\ \text{C}-\text{C}-\text{C} \\   \\ \text{N} \end{array}</math> </p>	0.28	1.7	-	1	-	+50
1-Hexylamine.....	<p style="text-align: center;"> <math>\begin{array}{c} \text{C} \\   \\ \text{C}-\text{C} \\   \\ \text{C}-\text{C}-\text{C} \\   \\ \text{N} \end{array}</math> </p>	0.77	1.9	-	1.7	-	+100
2-Hexylamine....	<p style="text-align: center;"> <math>\begin{array}{c} \text{C}-\text{C}-\text{C}-\text{C} \\   \\ \text{C}-\text{C} \\   \\ \text{N} \end{array}</math> </p>	0.90	2.2	-	2	-	+70
3-Methyl-1-hexylamine	<p style="text-align: center;"> <math>\begin{array}{c} \text{C} \\   \\ \text{C}-\text{C} \\   \\ \text{C}-\text{C}-\text{C} \\   \\ \text{N} \end{array}</math> </p>	0.11	2.6	-	0.7	-	+70
Amphetamine.....	<p style="text-align: center;"> <math>\begin{array}{c} \text{C}=\text{C} \\    \\ \text{C}-\text{C} \\   \\ \text{C}=\text{C} \\   \\ \text{C}-\text{C}-\text{C}-\text{C} \\   \\ \text{N} \end{array}</math> </p>	-	4.2	4.5	8	7.5	+50

of potency of the compounds, 4-methyl-2-hexylamine most potent, 2-heptylamine next, 5-methyl-2-hexylamine next and so on, is nearly the same for all three procedures.

Gurd (10) has suggested that the duration of action of a sympathomimetic amine is probably just as important, if not more so, than its absolute potency. Some attempts were made to measure the area under the pressor response curve which he suggested as a possible method, but were abandoned when it was found that many preparations did not return to the pre-injection pressure level during the course of an experiment. As an approximation, the time that the blood pressure was elevated over one half the total increase in pressure was used (in fig. 1 for example, the perpendicular distance A-A is bisected, a line drawn parallel to the base line through this center point, and the distance D converted to time). The average "50 per cent duration times" are given in table 1.

**ISOLATED TISSUE SEGMENTS.** 1. *Methods.* Sections of jejunum from 5 rabbits were placed in oxygenated Tyrode solution at 37-38°C. After some preliminary observations, 2 mgm. amine sulfate per 50 cc. tissue bath was chosen as a standard concentration ( $2.5 \times 10^{-4}$  Molar in ammonium ion). After two minutes exposure to the drug, the bath was flushed out three to five times. The responsiveness of the segments to 12.5 micrograms epinephrine base was used as a control. In a similar manner, virgin albino rat uterine horns in non-oxygenated, glucose-free Tyrode solutions were exposed to 10 mgm. of amine sulfates per 50 cc. bath. The relative antagonism of this dose to 75 micrograms epinephrine was determined. Sections of ileum of five guinea pigs were suspended in oxygenated Tyrode solution and 5 micrograms histamine base added to the 50 cc. bath. Two minutes later 2 mgm. amine sulfate were added and the effects noted after an additional two minutes. Four cat hearts were prepared for perfusion by the Langendorff method and the agents added to the perfusion fluid in amounts calculated to give concentrations of 1:10,000-1:50,000. Four guinea pig lungs were perfused by the method of Tainter, Pedden, and James (11). Following constriction with 1 mgm. histamine, 1 to 10 mgm. of these amines were administered.

2. *Results.* Experiments with these agents on isolated tissues are discouraging since the compounds are not particularly active. The effects on isolated rabbit jejunum are summarized in table 1. Lower concentrations seldom had a reproducible effect, and higher concentrations usually produced the opposite effect, a diminution in tone and amplitude which was not always reversible. Similarly, Alles (12) found that these higher concentrations ( $10^{-3}$  Molal) of 2-heptylamine produced relaxation and diminution of tone of isolated rabbit ileum, and that this dose antagonized both  $10^{-6}$  Molal epinephrine and acetylcholine. There is apparently no obvious correlation between the quantitative contractile effect on rabbit gut and that on the vasopressor system.

Adequate doses of these agents always contracted the rat uterus. The contractile activity was sufficient to antagonize the relaxant action of the epinephrine. The agents were all approximately of the same order of activity with the 1-hexylamine and 1-heptylamine appearing to be the most potent. Conversely, these agents relax guinea pig ileum contracted with histamine, although again 1-heptylamine was most potent, and the other agents of about half its activity, except 3-heptylamine and 4-heptylamine which were virtually inactive.

The concentrations used for the perfused heart usually produced only decrease in rate, force of contraction, and amount of outflowing perfusate. Occasionally, a slight preliminary increase in rate was observed, but this was usually not reproduced by the same concentration of the same amine in the same heart preparation. The agents did not antagonize the histamine constriction of the perfused guinea pig lung.

**ACTION IN MAN.** A young adult male weighing 79 kgm. received 2 mgm. of the sulfate salts of the three most active agents per kgm. two and one-half hours after a light morning meal. The agent was taken orally with 200 cc. warm water. The systolic and diastolic blood pressure and the pulse rate were recorded every 15 minutes for 3 hours while the subject remained sitting quietly. Experiments were performed once each week. The blood pressure slowly rose from a normal of 112/68 (70) to 134/90 (60) in 90 minutes after the ingestion of 4-methyl-2-hexylamine, from 110/70 (72) to 126/86 (64) in 90 minutes after 2-heptylamine, and from 110/70 (70) to 118/76 (68) in 90 minutes after 5-methyl-2-hexylamine.

The blood pressure and pulse rate began to return to normal after 150-180 minutes. Three mgm. doses of these agents per kgm. were not well tolerated by the subject who complained of dry mouth, palpitation, head-ache, numbness of extremities, and exaggerated pilomotor response. The blood pressure rose to higher levels, but these were irregular, as was the pulse rate. Three-tenths mgm. amphetamine sulfate per kgm. produced blood pressure changes similar to 2 mgm. 4-methyl-2-hexylamine sulfate, but with the addition of the usual central nervous system stimulant effects.

**DISCUSSION.** Examination of the structural formulas of these agents (see table 1) leads to certain comments. 4-Methyl-2-hexylamine and 2-heptylamine can be written with the carbon atoms in such positions that the compounds appear as fragments of the amphetamine or phenisopropylamine molecule. With no restricted rotation about the carbon-carbon bonds in these compounds, these compounds undoubtedly do not always possess this structural relationship to the amphetamine molecule. However, these are the only two compounds that can, under any circumstances, possess this space relationship, and are the only agents which have any similar vasopressor activity. 5-Methyl-2-hexylamine might be likened to an ortho-methylamphetamine, about which little is known, and 3-methyl-2-hexylamine is similar to beta-phenyl-n-propylamine, which is known to be less potent than amphetamine (13). One is tempted to conclude that some specific size and shape relationships exist for the cardiovascular sympathetic effectors; that the surface topology is such that agents that exceed acceptable dimensions in certain directions cannot be effective, and that conversely, if the shape does not exceed certain minimum specifications little activity will result. This is obviously just a restatement of Ehrlich's "lock and key" and Clark's "active patch" concept of drug action (14). However, 1-hexylamine and 1-heptylamine are relatively inactive, although they are the obvious relatives of phenethylamine, which is quite active under the conditions of this type of vasopressor assay (1). The duration relationships do seem to agree with some similar type of concept. The 4-methyl-2-hexylamine and 2-heptylamine have a long duration of action similar to that of amphetamine; the 1-hexylamine, 3-methyl-1-hexylamine, and 1-heptylamine have a much shorter duration of action which is more characteristic of the phenethylamine type molecule.

With isolated tissue segments, this specific relationship between chemical structure and physiological action disappears, for these agents are very similar in action as long as the amino group is not too far from the end of an alkane chain. Also, the concentrations needed to show a demonstrable effect are very large, and it is possible that surface tension and other ionic effects which are not related to autonomic effector cells play the predominant role.

Although some tachyphylaxis occurs with these agents, it is presumably not of great importance since considerable effect still is found after only short time intervals (fig. 1) and virtually no change in response is found after a 24-hour rest period. These agents must either be poorly absorbed from the human gastrointestinal tract, or else are subject to considerable destruction during absorption.

## SUMMARY

1. The vasopressor activity of the isomeric heptylamines in anesthetized and unanesthetized dogs has been determined and compared with epinephrine. The compounds range from agents with very short duration of action and almost no pressor action to 4-methyl-2-hexylamine which is about 1/200 as active as epinephrine and has a long duration of action.
2. The heptylamines, with the exception of the 4-heptylamine which was inactive, produced an increase in tone in the isolated rabbit jejunum with a concentration of 4 mgm. per 100 cc.
3. In adequate doses, the heptylamines caused contraction of the rat uterus and antagonized the relaxant action of epinephrine.
4. In the perfused heart, the agents produce a decrease in rate, decrease in force of contraction, and decrease in outflow of perfusate.
5. The heptylamines do not antagonize histamine constriction in the perfused guinea pig lung.
6. Orally, in man, the heptylamines have but little pressor action.

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# ANTIBIOTIC LACTONES AND SYNTHETIC ANALOGS

## I. CARDIOTOXIC EFFECTS ON THE ISOLATED FROG HEART<sup>1, 2</sup>

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The pharmacologic function of the lactone structure is of a wide diversity. Early investigators took advantage of its toxic properties in the use of lactones as insecticides (1). Lactones have been tried experimentally as anthelmintic agents (2). The unsaturated lactone ring of the cardiac glycosides has been recognized as an essential moiety for the cardiotonic action of these substances (3). The current researches on antibiotics have revealed a widespread occurrence of the lactone configuration among antibiotic substances from fungi (4), lichens (5), and higher plants (6). Recently, there have appeared reports of the value of lactones in the chemotherapy of experimental trypanosomiasis (7, 8), influenza virus infection (9), and certain bacterial infections (10).

Such findings suggest the possibility that these agents and similar substances may be of some clinical importance in the future, in which event the need for a study of their cardiac toxicity, among other toxic manifestations, is apparent. At the same time, however, it is felt that a comparison of the cardiotoxic effects of these agents with those of the cardiac glycosides might reveal a potentiality for cardiotonic activity among them. This activity, observed more directly (i.e., on the hypodynamic heart), will be reported in a subsequent work.

It is the purpose of this investigation, therefore, to examine the nature of the toxic effects on the isolated frog heart of several lactone derivatives, some of which have been shown to possess antibacterial, antiviral, antiprotozoan, and other important pharmacologic activity. An attempt will be made to relate chemical structure with cardiac toxicity.

In most instances the compounds tested were generously contributed by several industrial research institutes and academic laboratories. These sources are indicated in table I, where all of the agents appear.

**METHODS.** All of the experiments were carried out on male and female frogs of the species *Rana pipiens* in September, October, and November of 1947.

The cannula used throughout was of the same type as that described by Krayer (11). The fluid in the cannula was continuously replaced at the rate of 2.0 to 2.5 cc. per minute by allowing fresh solution to drop into it from a Mariotte bottle. The overflow side-arm

<sup>1</sup> The material presented here represents a portion of the data contained in a dissertation submitted on May 1, 1948 to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

<sup>2</sup> Aided in part by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

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of the cannula made it possible to maintain a constant hydrostatic pressure of 10 cm. of water on the heart, which was cannulated through the left branch of the aorta and attached to a light, well-balanced lever for recording ventricular excursions on a vertical kymograph. It should be noted that this pressure on the ventricle does not exceed the systolic pressure in the intact frog which has been calculated as 30 to 50 cm. of water (12). The importance of a constant initial tension has been emphasized by Wiggers, who has concluded that the analysis of changes in depressed hearts (i.e., depressed by certain drugs) is greatly influenced by the initial tension and by the initial fiber length (13).

The perfusion fluid was the same as that used by Krayer and had the following composition: NaCl, 0.65 per cent; KCl, 0.014 per cent; CaCl<sub>2</sub>, 0.011 per cent; and NaHCO<sub>3</sub>, 0.02 per cent.

All of the compounds were dissolved in 95 per cent ethyl alcohol to make up stock solutions varying from 1 per cent to 5 per cent. Appropriate dilutions of these solutions were made prior to each experiment and at no time did the alcohol concentration exceed 0.1 per cent. Several control experiments served to show that this concentration of alcohol produced no detectable toxic changes in the heart during the experimental observation periods.

The pH of all solutions was adjusted to that of the perfusion fluid, which was maintained between pH 7.5 and pH 7.9. This precaution is optional since Clark has shown that a pH as alkaline as 8.5 did not injure the isolated frog heart and that a pH as acidic as 6.4 was necessary before any injurious effect could be observed (14).

The experiments were carried out at room temperature which varied between 23°C. and 25°C. The preparation was surrounded with a suitable cylinder of heavy cellophane to protect it from sudden changes in temperature.

The method employed in these experiments for studying cardiac toxicity consisted of perfusing the heart with Ringers solution until the rate and amplitude of contraction became constant, at which time the chemical agent dissolved in Ringers solution was started through the heart and continued to the point of cardiac standstill. The normal isolated frog heart perfused in this manner with Ringers solution alone will continue to beat forcefully for 24 to 48 hours.

**RESULTS.** A tabulation of the time required for cardiac standstill at various concentrations and a characterization of the toxic effects appears in table I.

**1. Lactone series.** The saturated lactones, gamma-valerolactone, butyrolactone, and delta-caprolactone, displayed a comparatively low order of cardiac toxicity. At the high concentration of 100 mgm. per cent<sup>4</sup> these compounds caused cardiac standstill in 300 to 525 minutes, while their unsaturated analogs induced standstill in 25 to 110 minutes at the same concentration. Another fundamental difference between the saturated and unsaturated lactones existed in the type of standstill which supervened, for while the unsaturated compounds produced standstill in systole (the typical digitalis effect), the saturated derivatives always stopped the heart in diastole. At the time of diastolic standstill, moreover, the auricles continued to beat forcefully for as long as one hour after the ventricle had ceased. All three of the saturated compounds occasioned similar effects on ventricular rate and amplitude: the rate progressively decreasing, and the amplitude initially increasing, because of greater relaxation in diastole, and then gradually decreasing until the heart ceased its normal functioning.

While no attempt was made to study the quantitative aspects of the time-

<sup>4</sup>The term mgm. per cent used here and hereafter is meant to imply the concentration of the chemical agent in milligrams per 100 cc. of perfusion fluid.

TABLE I  
*Toxicity of various lactones and related compounds on the isolated frog heart*

COMPOUND (SOURCE)	CHEMICAL STRUCTURE	NUMBER HEARTS	CONC'N m.m. %	MEAN TIME TO CARDIAC ARREST min.	REMARKS
Butyrolactone (Cliffs-Dow Chem. Co.)	$\begin{array}{c} \text{H}_3\text{C}-\text{CH}_2 \\   \\ \text{H}_3\text{C}-\text{C}=\text{O} \\   \\ \text{O} \end{array}$	4	10.00	498	Heart stopped in diastole
Gamma-valerolactone (Mon- santo Chem. Co.)	$\begin{array}{c} \text{H}_3\text{C}-\text{CH}_2 \\   \\ \text{H}_3\text{C}-\text{CH}-\text{C}=\text{O} \\   \\ \text{O} \end{array}$	4	10.00	485	Heart stopped in diastole
Delta-caprolactone (Calco Div. Am. Cyanamid Co.)	$\begin{array}{c} \text{CH}_2 \\ \diagdown \\ \text{H}_3\text{C}-\text{CH}-\text{C}=\text{O} \\   \\ \text{CH}_2 \\ \diagup \\ \text{H}_3\text{C} \end{array}$	4	10.00	292	Heart stopped in diastole
2-pentene-1,4-oxide* (Ster- ling-Winthrop Res. Inst. & Calco Div. Am. Cyanamid Co.)	$\begin{array}{c} \text{HO}=\text{CH} \\   \\ \text{H}_3\text{C}-\text{CH}-\text{C}=\text{O} \\   \\ \text{O} \end{array}$	3	10.00	68	Systolic standstill; gradual decrease in rate
		3	2.00	90	
		3	1.00	215	
		3	.20	349	
		3	.10	612	

3-pentene-1, t-alidol (Sterling-Winthrop Res. Inst. & Calco Div. Am. Cyanamid Co.)		2 3 4 4 3 2	10.00 2.00 1.00 .20 .10 .05	18 41 110 162 210 537	Systolic standstill preceded by pronounced increase in amplitude; gradual increase in rate
Dilactone of pulvinic acid (Dr. W. Bergmann, Yale Chem. Dept.)		8 4 4 4 2	.04 .02 .01 .005 .002	15 22 38 110 600	Systolic standstill preceded by prolonged increase in amplitude
Clavacin (Vick Chem. Co. & Dr. H. Raistrick, Lond. Sch. Hyg. & Trop. Med.)		3 4	10.00 5.00	21 180	Systolic standstill preceded by prolonged increase in amplitude
Isochavacin (Vick Chem. Co.)		4	10.00	110	

TABLE I—Continued

COMPOUND (SOURCE)	CHEMICAL STRUCTURE	NUMBER HEARTS	CONC'N mgm. %	MEAN TIME TO CARDIAC ARREST	REMARKS
Dimethylisoclavacin (Vick Chem. Co.)	<p>Chemical structure of Dimethylisoclavacin: A bicyclic lactone with two methyl groups at the 2-position.</p>	4	10.00	min. 204	
Penicillio acid (Dr. H. Rais-trick, Lond. Sch. Hyg. & Trop. Med.)	<p>Chemical structure of Penicillio acid: A penicillin-like molecule with a methoxy group at the 6-position.</p>	3	10.00	87	
Carolic acid (Dr. H. Rais-trick, Lond. Sch. Hyg. & Trop. Med.)	<p>Chemical structure of Carolic acid: A penicillin-like molecule with a hydroxyl group at the 6-position.</p>	3	10.00	195	
Terrestric acid (Dr. H. Rais-trick, Lond. Sch. Hyg. & Trop. Med.)	<p>Chemical structure of Terrestric acid: A penicillin-like molecule with a hydroxymethyl group at the 6-position.</p>	3	10.00	262	

			6 5	10.00 1.00	110 215	Initial increase in amplitude
Parisorbic acid (Calco Div. Am. Cyanamid Co.)						
Coumarin (Dr. W. Bergmann, Yale Chem. Dept.)		3	10.00	227		Pronounced increase in rate
Coumalinic acid (Sterling-Winthrop Res. Inst.)		3	10.00	45		
Meconic acid (Dr. W. Bergmann, Yale Chem. Dept.)		4	10.00	30		

TABLE I—Continued

COMPOUND (SOURCE)	CHEMICAL STRUCTURE	NUMBER HEARTS	CONC'N mgm. %	MEAN TIME TO CARDIAC ARREST min.	REMARK
Kojic acid (Northern Reg. Res. Lab.)		3	10.00	205	
Furfural (Quaker Oats Co.)		3	10.00	165	Heart stopped in diastole
Furfuryl alcohol (Quaker Oats Co.)		3	10.00	150	Heart stopped in diastole
Nitrosurazone (Eaton Labs., Inc.)		4	.80	—	No standstill after per- fusion for 400-600 min- utes

\* Delta-1 angelicalactone.  
† Delta-2 angelicalactone.

concentration relationships of the saturated lactones, it may be observed from table I, nevertheless, that the six-membered heterocyclic structure of the delta-caprolactone proved more toxic than the two five-membered gamma-lactones.

The most cardiotoxic agents in this study were found among the unsaturated lactones. Three of these were chosen for an investigation of their time-concentration relationships in comparison to those of ouabain and digitoxin. The usual hyperbolic function of log. concentration against time was readily converted to a straight line function by plotting log. concentration against log. time (fig. 1). If non-parallelism is disregarded, it may be observed by extrapolation that the 3-pentene-1,4-oxide is roughly 10 times more toxic than its bond isomer, 2-pentene-1,4-oxide, a conclusion reached by Mendez in an earlier work (15). Furthermore, it appears from figure 1 that the dilactone of pulvinic acid is of a

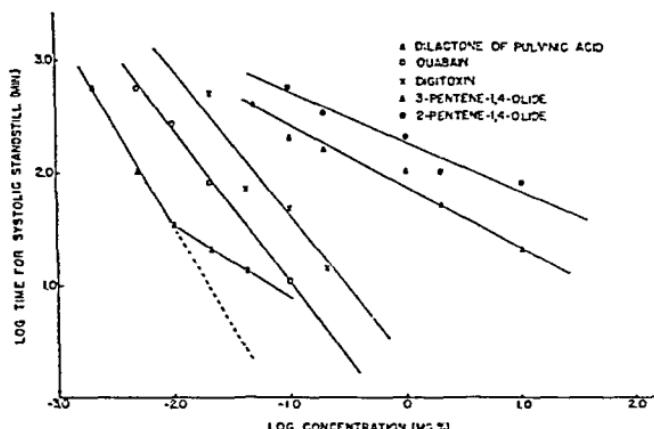


FIG. 1. CONCENTRATION-TIME RELATIONSHIPS FOR THE CARDIOTOXIC ACTION OF CERTAIN LACTONES AND CARDIAC GLYCOSIDES

The comparatively sharp and early break in the dilactone curve might possibly be a reflection of some characteristic physical property, e.g., the degree of dissociation.

higher order of toxicity than either of the cardiac glycosides. The digitalis-like systolic standstill produced by this compound may be seen in figure 2.

It seemed important, in the light of the high cardiotoxicity of the dilactone of pulvinic acid, to make a precise measurement of its relative toxicity. This was accomplished by analyzing statistically the dosage-mortality data obtained from small numbers of hearts according to the method of maximum likelihood (16). The end-point in these studies was systolic standstill, but with the time restriction that the standstill had to occur within 120 minutes to be included in the data. Table II summarizes the results.

The relatively high standard errors, which range from 20 to 30 per cent, may be explained on the basis of the small samples of population tested. It should be pointed out that these comparisons involve concentrations in micrograms per cent; recalculation on a millimolar basis<sup>4</sup> gives a ratio of 1:2.4 for the dilactone.

<sup>4</sup> The following molecular weights were used: ouabain = 584; dilactone of pulvinic acid = 290.

The clavacin series provided an interesting relation between chemical structure and cardiotoxic action. The most toxic agent proved to be clavacin, which contains a double bond in the delta-2 position of its lactone ring. The effect of 5 mgm. per cent was characterized by an initial pronounced increase in amplitude, usually more than 25 per cent of the normal maximum contraction, which persisted for long periods of time (up to 60 minutes)—cf. fig. 3. Thus the toxic action of clavacin on the frog heart is preceded by a prolonged positive inotropic

## 36

$\uparrow$   
MICROGRAMS %  
PULVINIC ACID

FIG. 2. THE TOXIC ACTION OF THE DILACTONE OF PULVINIC ACID ON THE ISOLATED FROG HEART

Time is recorded in 5 second intervals; the number above the time interval line indicates heart rate per minute (the initial rate for this particular heart was 44).

TABLE II

*Relative cardiototoxicity of ouabain, digitoxin, and the dilactone of pulvinic acid*

DRUG	LD-50 $\pm$ S.E.	REL. POTENCY $\pm$ S.E.	POTENCY RATIO	NO. HEARTS
	%			
Ouabain	37.3 $\pm$ 7.2	—	1	17
Digitoxin	83.8 $\pm$ 15.9	.445 $\pm$ .12	1:2.3	21
Dilactone of pulvinic acid	44.5 $\pm$ 8.6	.836 $\pm$ .22	1:1.2	18

effect. Isoclavacin, on the other hand, with its double bond in the delta-1 position, demonstrated roughly one-tenth the toxicity of clavacin, a situation which bears a striking similarity to the cardiotoxic potencies of the angelicalactones (i.e., the 2- and 3-pentene-1,4-olide). The addition of methyl groups to isooclavacin, as in dimethylisooclavacin, produced an even greater diminution in activity.

In the unsaturated delta-lactone series (characterized by an increase of one carbon atom over the five-membered ring of the unsaturated gamma-lactones), the most notable feature has been a general decrease in activity. The most

toxic agent in this group was coumalinic acid, a compound related to parasorbic acid, and containing a series of conjugated double bonds. Parasorbic acid, however, was the only drug of the series to induce an initial increase in the amplitude of ventricular contraction before the ultimate systolic standstill. Coumarin, on the other hand, while devoid of an appreciable action on the amplitude of ventricular contraction, demonstrated a pronounced stimulation of cardiac rate at a concentration of 20 mgm. per cent.

*2. Gamma-pyrone series.* These compounds differ from the lactones in that the carbonyl group has been displaced from an ortho orientation with the oxygen bridge to a para orientation with it. The two agents studied, kojic and meconic acids, demonstrated an order of cardiac toxicity which was unexpectedly high, since the gamma-pyrone structure does not possess the characteristic chemical properties of the lactones. The more potent, meconic acid, which also possesses a series of conjugated double bonds, approached the activity of 3-pentene-1, 4-olide. Kojic acid, although much less toxic, still caused ultimate systolic standstill. A concentration of 40 mgm. per cent produced an initial increase in ampli-

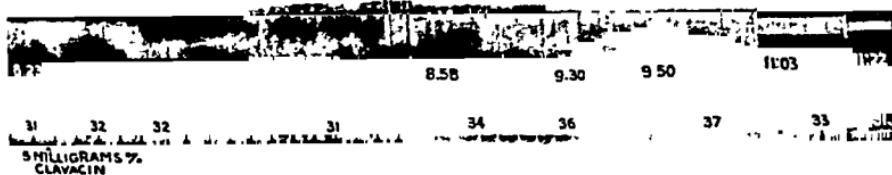


FIG. 3. THE TOXIC ACTION OF CLAVACIN ON THE ISOLATED FROG HEART  
The numbers directly below the tracing represent time of day; the numbers above the time interval line indicate heart rate per minute; time is recorded in 5 second intervals.

tude and the eventual development of bigeminal rhythm and ventricular premature contractions.

*3. Furan series.* The furan structure is more of a departure from the lactone configuration than the gamma-pyrone structure, because furans do not contain a carbonyl group, and retain only the oxygen bridge of the lactones. The three derivatives studied, furfural, furfuryl alcohol, and 5-nitro-2-furfuraldehyde semicarbazone<sup>6</sup> (nitrosurazone), displayed a notably low order of cardiac toxicity. At a concentration of 0.8 mgm. per cent (the highest used because of solubility limits), the highly active antibacterial and antiprotozoan agent, nitrosurazone, produced no appreciable changes in ventricular contraction or rate, when perfused through the heart for periods up to five hours. The effects of furfural and furfuryl alcohol were indistinguishable from each other, both compounds causing increased diastolic relaxation and ultimate depression of the junctional tissue with resultant heart block and diastolic standstill, at concentrations of 10 to 20 mgm. per cent.

**DISCUSSION.** The higher activity of the delta-2 position of unsaturation in

\* This material is marketed by the Eaton Laboratories, Inc., Norwich, N. Y., under the trade name Furacin.

the angelicalactones is of special interest in relation to the unsettled problem of the position of the double bond in such highly active cardiac lactones as digitoxin and ouabain. The delta-2 position was originally established as the correct one by Jacobs, and later, by Windaus, Tschesche, and others (17). Elderfield, however, has recently presented important evidence pointing toward the delta-1 configuration (18).

The data presented here for the angelicalactones and the clavacin series seem to indicate the delta-2 position as that producing the higher activity. Still, the matter is confused by the outstanding potency of the dilactone of pulvinic acid, composed of two condensed gamma-lactone rings, both of which contain a double bond in the delta-1 position. The explanation for this seemingly confused state of facts may lie in subtle differences in chemical structure and, therefore, in chemical reactivity. The delta-2 angelicalactone (3-pentene-1,4-oxide) is characterized by a methylene group which is flanked by a vinyl and a carboalkoxy group, imparting a somewhat higher degree of activity than the delta-1 isomer (19). The vinyl group occupies such a position that the delta-2 angelicalactone might be regarded as the inner ester of an hydroxy-substituted vinyl acetate, thus:  $\text{C}-\text{C}=\text{CH}-\text{COOH}$ . The dilactone of pulvinic acid, on the other



hand, might be viewed as having been formed from a substituted butadiene, with its system of highly reactive, conjugated double bonds, thus:  $-\text{C}=\text{C}-\text{C}=\text{C}-$ .



It seems reasonable to infer, therefore, that while delta-2 unsaturation is most favorable for cardiototoxic activity in the simple lactone structure, still the delta-1 configuration can produce a high order of activity if incorporated in a system of conjugated double bonds.

The high activity inherent in the conjugated double bond system among these agents was evidenced further in the effects of coumalinic acid and meconic acid.

Effects displayed by the clavacin series are complicated by the fact that chemical reactivity in this group reflects a summation of gamma-pyrone and lactone reactivities. The ten-fold higher cardiototoxicity displayed by clavacin (delta-2) over its bond isomer, isoclavacin, is clear indication that the lactone moiety has a function in the degree of activity. On the other hand, the relative decrease in activity by methylation of the gamma-pyrone ring (isoclavacin to dimethylisoclavacin) certainly points toward a pyrone function in the cardio-toxicity. This latter view is borne out by the relatively high toxicity of meconic acid, which is a true gamma-pyrone, uncomplicated by other ring systems.

#### CONCLUSIONS

1. Twenty lactone antibiotics and synthetic analogs have been studied for their cardiotoxic actions on the isolated frog heart.
2. The dilactone of pulvinic acid produced systolic standstill in the same order of concentration as that necessary for the cardiac glycosides, ouabain and digitoxin, to produce the same toxic effect.

3. Among the lactones, delta-2 unsaturation appeared more favorable for cardiotoxic action than delta-1 unsaturation, except when the latter was incorporated in a system of conjugated double bonds.

4. Alkylation and hydrogenation of the lactones led to a decrease in activity.

5. The gamma-pyrone structure, when containing a system of conjugated double bonds, showed an order of activity similar to the unsaturated lactones.

6. The furan structure and the saturated lactone configuration demonstrated a low order of cardiotoxicity and failed to induce systolic standstill.

The author wishes to acknowledge his indebtedness to the following for having made available many of the compounds tested: Dr W. Bergmann, Department of Chemistry, Yale University; Dr. M. L Tainter, Sterling-Winthrop Research Institute, Dr M. L Crossley, American Cyanamid Company, Calco Division.

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# INFLUENCE OF TETRAETHYLMONIUM ON RESPONSES OF ISOLATED INTESTINE TO ANGIOTONIN AND OTHER SUBSTANCES

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It has been reported by Page and Taylor (1) and Page (2) that the tetraethylammonium ion increases responses of blood pressure to angiotonin (hypertensin) and also to various other substances (e.g., histamine, barium chloride and mecholyl). The question arose whether this phenomenon is exhibited by excised tissue and by any effectors other than those involved in blood pressure responses. Therefore, a study was made of the effect of the presence of the tetraethylammonium ion on the responses of isolated intestine to angiotonin, histamine, barium chloride and acetylcholine. An abstract of this work has appeared (3).

**MATERIALS AND METHODS** The terminal ileum of the fasted male guinea pig (weighing about 300 grams), freshly killed by a blow on the head and exsanguinated by decapitation, was used. After flushing out the lumen with Tyrode's solution a segment was suspended, with both ends open, in oxygenated Tyrode's solution in a muscle chamber of 6 cc capacity. Contractions were recorded by a constant pressure lever magnifying 9 times. The length of the muscle was 4.0-4.5 cm when loaded and at 38°C. Solutions of angiotonin and other substances were introduced under the surface of the Tyrode's solution in the chamber with a syringe. Previously a volume equal to that of the solution to be introduced was withdrawn from the muscle chamber. After a maximal contraction was obtained, the gut was washed without exposure to air. Tyrode's solution, kept under constant pressure in a reservoir, was allowed to flow into the muscle chamber through a side tube near the bottom and was simultaneously drawn off through a second side tube near the top by gentle suction into a graduate. The washing volume was 50 cc. To avoid temperature changes the muscle chamber, the reservoir for the Tyrode's solution, and the tube leading from the reservoir to the muscle chamber were all immersed in a constant temperature bath at 38°C. A rigid time schedule was adhered to in each experiment, fixed times being allowed for contraction and rest. The times varied with the substance being investigated.

In determining the effect of the tetraethylammonium ion on responses to angiotonin and the other spasmogenic agents, the tetraethylammonium was introduced into the muscle chamber during the rest period and thus was present when the spasmogenic agents were given.

Tetraethylammonium bromide was used in most of the experiments in the following three final dilutions 1:13,000, 1:20,000 and 1:40,000. In some instances the chloride was used in equivalent concentrations with similar results.

Angiotonin was prepared from heavily heparinized dog's plasma. An excess of renin<sup>1</sup> was added to the plasma, and the mixture was incubated for 6 minutes at 38°C. It was immediately brought to about pH 5 with 1.0 N HCl and then heated at 90°C with stirring for 6 minutes. The fluid obtained on centrifugation was purified by adsorption procedures. The pH was brought to 6.9-7.0 (glass electrode) with NaOH and HCl, tribasic calcium phosphate in the amount of 20 mgm per cc was added, the mixture was stirred 8 minutes, and

<sup>1</sup> I am greatly indebted to Mr Alfred Barol and Dr F. W. Bernhart of the Wyeth Institute of Applied Biochemistry for supplying the renin used in these experiments.

the phosphate was removed by centrifugation and discarded. While the tribasic calcium phosphate adsorbs and thus removes some of the impurities, it does not adsorb an appreciable amount of angiotonin. Additional purification was accomplished by adsorption of the angiotonin on charcoal. The commercial charcoal, Darco, grade S-51, washed with 10 per cent HCl and then with distilled water and dried, was added to the fluid (40 mgm. per 1.0 cc.). After stirring for 10 minutes, the mixture was centrifuged and the fluid was discarded. The angiotonin was eluted from the charcoal by glacial acetic acid. One cc. of the acid per 40 mgm. of charcoal was added, the mixture was stirred 10 minutes, and the acid decanted and saved. The charcoal was washed similarly with 0.75 cc. and then with 0.5 cc. of acid per 40 mgm. of charcoal. The three acid washings were pooled. The angiotonin, now contained in the glacial acetic acid, was precipitated by the addition of 8 volumes of cold absolute diethyl ether. After standing in the refrigerator for 20 minutes and after centrifugation, the ether was decanted off and discarded. The precipitate was washed again with 3 volumes of cold ether, and was stored in a vacuum desiccator. Shortly before using, the material was dissolved in Tyrode's solution. Ordinarily the equivalent of a few hundredths of a cc. of the fluid resulting after heat coagulation serves to give a good response from the ileum. This amount contains about 0.01 unit of angiotonin (unit of Braun-Menéndez, Fasciolo, Leloir and Muñoz (4)). The only appreciable loss of angiotonin activity in the above preparation occurs in the charcoal procedure where a loss of roughly 40 per cent occurs.

**RESULTS.** The tetraethylammonium ion itself usually elicited some response (e.g., figure 1A). The contraction had usually subsided by the time the spasmodic agent was given.

*A. Angiotonin.* Responses to the angiotonin preparations were consistently increased by the presence of tetraethylammonium. This augmentation occurred in every test made, in 38 tests on 19 different muscles. Figure 1A shows one of these tests. A constant amount of angiotonin was given every 6 minutes at "A". The muscle was washed 3 minutes later. At the points marked "TEA", one minute after washing, tetraethylammonium in a final dilution of 1: 20,000 was given and was present in the bath when the angiotonin was given 2 minutes later. The two responses in the presence of tetraethylammonium are greater than those without this substance.

In about a third of the experiments atropine sulphate in a concentration of 1.0 microgram per 1.0 cc. was incorporated in the Tyrode's solution. Again responses to angiotonin were consistently augmented by tetraethylammonium.

The three concentrations of tetraethylammonium gave similar results qualitatively, but the augmentation tended to be greater with higher concentration.

Since angiotonin is not available in pure state, the possibility existed that the increased responses observed with tetraethylammonium might result from increased sensitivity to impurities contained in the preparation of angiotonin rather than to angiotonin itself. However, studies involving dummy or control preparations of angiotonin revealed that the activity due to impurities was by comparison extremely small. The control preparations were made from plasma and purified by adsorption by the same procedure used in the preparation of angiotonin except that heat inactivated renin was used. Hence no appreciable amount of angiotonin was present. Seven experiments were performed with these control preparations. Atropine sulphate, 1.0 microgram per cc., was incorporated in the Tyrode's solution. The doses of control preparations eliciting minimal

responses were determined. These threshold doses were 80 to 100 times greater than the amounts of angiotonin solution required to give responses of the usual

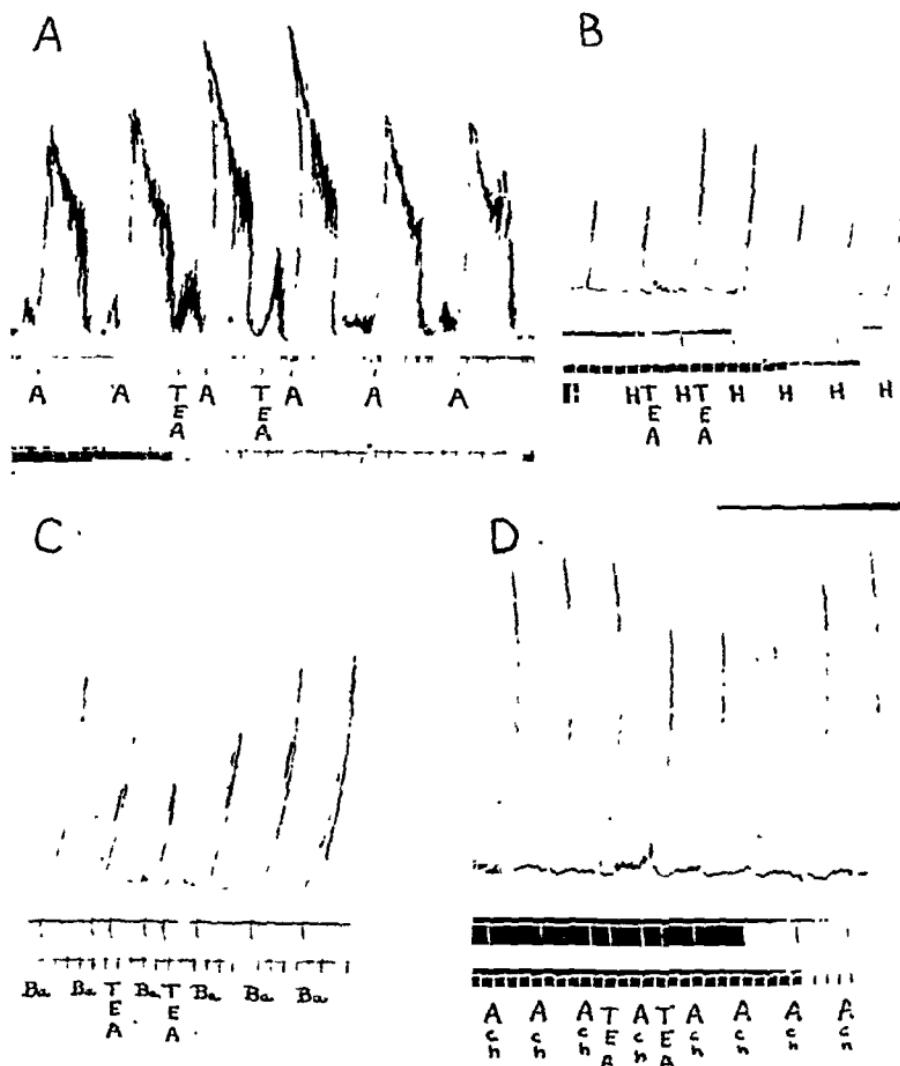


FIG. 1. The time interval on the bottom line is 1 minute. TEA = tetraethylammonium bromide in a final dilution in the muscle chamber of 1:20,000. A = 0.01 unit angiotonin. H = histamine diphosphate in a final concentration of 1:12 million in the muscle chamber. Ba = barium chloride in a final concentration of 1:10,000. Ach = Acetylcholine chloride in a final concentration of 1:50 million.

magnitude. It seems extremely unlikely that the augmentation by tetraethylammonium of responses from these angiotonin preparations could result from impurities which were present in amounts of about  $\frac{1}{80}$  of their threshold dose.

*B. Histamine.* In 21 tests on 10 muscles reactions to histamine diphosphate were uniformly increased by the tetraethylammonium ion (figure 1B). In general, the greater the concentration of tetraethylammonium, the more marked was the augmentation. No atropine was used in the Tyrode's solution in these experiments or with the remaining spasmogenic agents to be discussed.

*C. Barium chloride.* Tetraethylammonium usually depressed responses elicited by the barium ion. The tracing in figure 1C shows such a depression in responses to barium chloride in a final concentration of 1:10,000 in the muscle chamber. Depression occurred in 13 tests on 4 muscles. In only 1 muscle, in which 2 tests were made, did the tetraethylammonium ion increase the responses.

*D. Acetylcholine.* Responses to acetylcholine were usually depressed by tetraethylammonium. Acetylcholine chloride in a final dilution of 1:50 million was used in the test shown in figure 1D. Depression occurred in 19 tests on 10 muscles. In 10 tests on 3 muscles responses were augmented by the tetraethylammonium ion. Sometimes, i.e. in 8 tests involving both of the previously mentioned groups of muscles, no significant effect could be made out.

**DISCUSSION.** The effects of the tetraethylammonium ion on isolated intestine may be compared with those reported for blood pressure in the dog (1, 2). They may be construed as being analogous in the case of angiotonin and histamine and as usually opposite with barium and with acetylcholine. Tetraethylammonium increases both the spasmogenic intestinal action of angiotonin and its pressor effect in the dog. The contraction of the gut by histamine is intensified, and its action in lowering blood pressure is reported as usually being increased. However, the tetraethylammonium ion usually depresses responses to barium by the intestine but usually increases the responses to barium of the dog's blood pressure. The action of acetylcholine on the gut is usually decreased, while the effect of mecholyl on blood pressure is reported as usually augmented.

The concentrations of tetraethylammonium used in the case of the ileum approximate those employed in the studies involving blood pressure (2). In the latter instance, 100 mgm. of the chloride were given intravenously to dogs weighing about 10 kgm. Assuming distribution in extracellular fluid and an extracellular fluid volume of 3000 cc., the concentration of tetraethylammonium chloride is 1:30,000. The concentrations of the bromide used in the present study, when expressed on the basis of equivalent concentrations of the chloride, are roughly 1:16,500, 1:25,000 and 1:51,000.

These studies on the ileum may have a bearing on the interpretation of the data presented by Page and his collaborators (1, 2, 5). In describing the augmentation of responses in blood pressure by tetrachethylammonium, they have pointed out that destruction of the spinal cord, anterior rhizotomy, and ganglionectomy with subdiaphragmatic vagotomy likewise may increase blood pressure responses to angiotonin and other substances. Since there is evidence that the tetraethylammonium ion blockades autonomic ganglia (6), it might be inferred that the augmenting effect of tetrachethylammonium on responses in blood pressure is dependent on the interruption of the autonomic outflow from the central nerv-

ous system. This inference is invalid in the case of isolated intestine where the autonomic outflow has already been interrupted.

#### SUMMARY

The tetraethylammonium ion is capable of causing contraction of the isolated terminal ileum of the guinea pig.

The presence of this ion increases responses of the isolated ileum to angiotonin and histamine, and usually depresses those to barium chloride and acetylcholine.

**ACKNOWLEDGMENT.** The author is indebted to Betty Jayne Miller Angstadt, Shirley Friday and Marjory Gelpke White for their valuable technical assistance.

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# A NEUROLOGICAL SYNDROME INDUCED BY ADMINISTRATION OF SOME CHLORINATED TERTIARY AMINES<sup>1</sup>

A GOLDIN, H A NOE,<sup>2</sup> B H LANDING,<sup>3</sup> D M SHAPIRO,<sup>3</sup> AND  
B GOLDBERG

WITH THE TECHNICAL ASSISTANCE OF RUTH A FUGMANN, MARY A MILLER, AND  
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The nitrogen mustards have assumed an important role in investigations of tumor chemotherapy as well as in the various fields of fundamental biological research. A toxicity survey has been reported by Goldin, et al. (1) and the systemic pathology of a series of nitrogen mustards has been studied by Landing, et al. (2). During the course of these investigations, it was found that administration of two of the nitrogen mustards, namely, beta-chloroethyl-morpholine and beta-chloroethyl-dimethylamine induced pronounced changes in normal mice resulting in a behavior pattern similar to that exhibited by genetic Waltzer and Shaker strains. The injected animals exhibited hyperactivity, choreic head movements, retropulsion and had a tendency to run in circles. These effects were not produced by beta-hydroxyethyl-morpholine or by morpholine (3, 4). The current investigation was conducted to determine what compounds and chemical groupings among the nitrogen mustards are responsible for this effect and to obtain further information on the pharmacological action and histopathology resulting from the administration of these compounds.

**MATERIAL AND METHODS** Albino mice, Carworth Farms CF1 males, ranging in weight from 18-25 grams, two to three months of age, were used routinely in this study. In addition, CF1 females and C3H strain males and females were injected.

The compounds routinely were administered intraperitoneally in 0.9 per cent saline. Insoluble compounds were suspended in 10 per cent acacia. All solutions of chemical agents were prepared freshly prior to injection. In each instance, the total volume of agent plus diluent injected represented 1 per cent of the body weight of the animal. In an initial test for each compound, mice received a single injection of the agent at 5 geometrically spaced concentration levels ranging from 1 to 625 mgm /kgm. Nontoxic compounds (those

<sup>1</sup> This work was conducted in part by a grant from the American Cancer Society to the Department of Preventive Medicine, The Johns Hopkins University School of Medicine recommended by the Committee on Growth of the National Research Council. These studies form part of a joint project on the chemotherapy of cancer being conducted at The Johns Hopkins University School of Medicine, Department of Preventive Medicine and the Medical Division, Army Chemical Center, Maryland. Compounds were obtained from B Witten and A Reeves, Technical Command, Army Chemical Center, Maryland and the University of Chicago Toxicity Laboratory.

<sup>2</sup> Captain, M S C, U S Army

<sup>3</sup> Captain, M C, A US

producing no deaths up to 625 mgm./kgm.) were administered at higher concentration levels. In a second test phase, groups of mice were given a single injection at 5-6 geometrically spaced dosage levels, chosen in a narrower range, in the area of the median lethal dose ( $LD_{50}$ ) as estimated from the initial test. Saline- or vehicle-injected mice served as controls for each experiment. All surviving mice were observed for a minimum period of ten days for acute and chronic effects of the compounds. An approximation of the  $LD_{50}$  was obtained by plotting the data on log-probability paper.

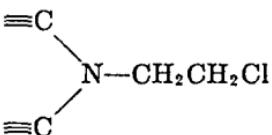
When the dosage levels used resulted only in 100 per cent and 0 per cent mortalities, supplementary experiments, at more closely spaced dosage increments, were conducted until intermediate percentage levels of death were obtained. This was done in order to insure observation of behavior pattern in survivors at a number of dosage levels at which toxic effects of the compound were manifested.

Criteria used as indicative of positive waltzing effect, referred to henceforth as the "waltzing syndrome", included hyperactivity, retropulsion, choreic head movement, running in circles, incoordination, poor balance, poor righting reflex and uncoordinated swimming pattern. These criteria are essentially the same as those described by Grüneberg (5) for genetic Waltzer and Shaker mice.

The central nervous systems of treated and control mice were fixed by removing the calvarium, and placing the base of the skull, with the brain *in situ* and the spinal column attached, in 10 per cent formalin. The brain and cord were dissected free after fixation, embedded in paraffin, and sectioned at 5 microns. For study of the inner ears, intact skulls were fixed in hyperacidified Zenker's solution. Additional decalcification, if necessary after fixation, was done in formic acid solution. Embedding was carried out as above. Hematoxylin and eosin, cresyl violet, and pyridine-silver staining procedures were used.

**EXPERIMENTAL.** 1. The compounds listed in table I elicited the waltzing syndrome in mice.

All of these compounds have the following nucleus:



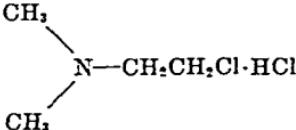
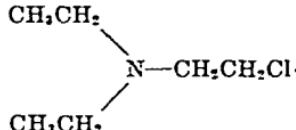
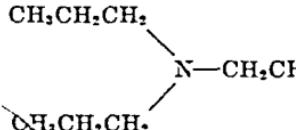
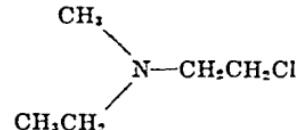
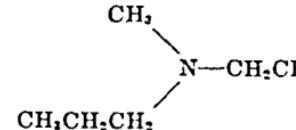
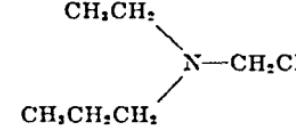
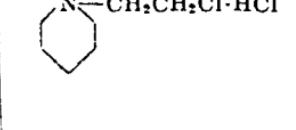
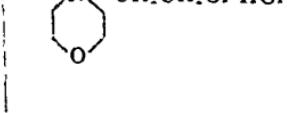
From the table it is apparent that the waltzing syndrome appeared at concentrations in the area of the median lethal dose. The symptoms appeared within six to twelve hours after injection and seemed to be fully developed by twenty-four hours. The induced effects were apparently permanent in nature. This is indicated by the fact that some of the treated mice, kept for as long as six months, showed no diminution of the waltzing pattern. Within the dosage range in which waltzing was produced there was a lessened intensity of effect as the concentration level was diminished.

The swim test appeared to be the most sensitive test for determining the waltzing effect and was particularly valuable where the behavior pattern appeared questionable. Incoordination in swimming, inability to remain above the surface of the water, swimming in a spiral pattern and swimming in circles were quite obvious in mice which otherwise exhibited the effect in only mild form. Normal mice swim in a well coordinated manner, usually in a straight line.

The inability of treated mice to maintain their balance when placed on a narrow horizontal bridge appeared to be the next most sensitive criterion. Normal mice maintain balance with no difficulty.

All of the compounds listed in table I appeared to produce the same type of

TABLE I  
*Beta-chloroethylamines which induced the waltzing syndrome*

CHEMICAL NAME	FORMULA	LD <sub>50</sub>	DOSAGE RANGE PRODUCING WALZING
Dimethyl-β-chloroethylamine·HCl		1.950	1.350-2.340
Diethyl-β-chloroethylamine·HCl		0.414	0.308-0.726
Dipropyl-β-chloroethylamine·HCl		0.351	0.320-0.465
Methyl-ethyl-β-chloroethylamine·HCl		1.300	1.050-1.870
Methyl-n-propyl-β-chloroethylamine·HCl		0.639	0.540-0.965
Ethyl-n-propyl-β-chloroethylamine·HCl		0.374	0.374-0.534
β-chloroethyl-piperidine·HCl		0.505	0.380-0.505
β-chloroethyl-morpholine·HCl		0.865	0.270-1.080

behavior pattern, and differed only in the range of concentration which produced the effect. The effectiveness of the compounds was not limited to Carworth Farms male mice, for comparable results were obtained with Carworth Farms females and C3H strain males and females.

The cumulative effect of seven of these compounds was tested by administering, intraperitoneally, daily doses at various fractions of the median lethal dose. Four mice were injected at each dosage level. The results are summarized in table II. The waltzer-producing compounds were apparently only mildly cumulative in their effects.

TABLE II  
*The effect of repeated administration of waltzer-inducing compounds*

COMPOUND	DOSAGE LEVEL	% OF LD <sub>50</sub>	NUMBER OF INJECTIONS	WALTZING SYNDROME
<i>mM/kgm.</i>				
Diethyl-β-chloroethylamine·HCl	0.207	50.0	7	-
Dipropyl-β-chloroethylamine·HCl	0.090	25.6	7	-
Methyl-ethyl-β-chloroethylamine·HCl	0.360	27.7	7	-
Methyl-n-propyl-β-chloroethylamine·HCl	0.320	50.0	7	-
Ethyl-propyl-β-chloroethylamine·HCl	0.086	22.9	7	±
β-chloroethyl-piperidine·HCl	0.253	50.1	5	+
	0.127	25.1	7	-
β-chloroethyl-morpholine·HCl	0.254	29.4	1	+
	0.128	14.8	10	+
	0.072	8.4	20	+
	0.051	5.9	20	-

2. The beta-chloroethylamine structure is characteristic of the nitrogen mustard compounds and forms an ethylenimonium cation in polar solvents. The high reactivity of this ring structure (6, 7, 8, 9) presumably is responsible for the characteristic biological actions of this group of compounds. In order to obtain evidence as to whether the =N-beta-chloroethyl structure is requisite for the production of the waltzing syndrome, a series of compounds was tested in which a hydroxyl group was substituted for the beta chlorine of compounds which produce waltzing. The hydroxyl group was chosen since the beta-hydroxy compound represents the terminal stage in the hydrolysis of the beta-chloro amines, and itself does not undergo cyclization (10). The results are summarized in table III, A. Failure of these hydroxy compounds to produce waltzing indicates an importance of the =N-beta-chloroethyl structure or the imonium moiety to the production of the waltzing syndrome.

3. To determine the effect of the completeness of alkylation of the mono-beta-chloroethylamine on the production of the waltzing syndrome, the primary amine, beta-chloroethylamine, and a series of mono-alkyl-beta-chloroethylamines were tested. The results are summarized in table III, B. None of these compounds produced the waltzing syndrome.

There is, thus, a qualitative difference in the behavior of mice after administration of primary and secondary halogenated amines as compared with tertiary beta-chloroethylamines. The specific waltzer-inducing action of beta-chloroethylamines is dependent not only on the presence of the beta-chloroethylamine structure and on the presence of alkyl substituents, but also on the number of alkyl groups attached to the nitrogen atom.

4. To test whether, in the dialkyl-beta-chloroethylamines, the dialkyl structure, *per se*, without regard to the nature of the substituents on the alkyl carbons, is a sufficient condition for the production of the waltzing syndrome, substituent groups were introduced into the dialkyl radicals, replacing hydrogen. As can be seen from table III, C, the substitution of two phenyl groups, two para-chlorophenyl groups, or two paramethoxyphenyl groups in dimethyl-beta-chloroethylamine is sufficient to change the specificity of biological action of the compounds so that no waltzing appears.

5. Since formation of the cyclic onium cation is presumably characteristic of beta-halogenated alkylamines, the ability of dimethyl-beta-bromoethylamine, the bromo analogue of the waltzer producing compound dimethyl-beta-chloroethylamine, was tested for its ability to produce waltzing. Another beta-bromo compound, phenyl-beta-bromoethylamine, was likewise tested. Reference to table III, D, shows that neither of these compounds produced the waltzing syndrome. Dimethyl-beta-bromoethylamine is considerably more toxic than dimethyl-beta-chloroethylamine.

6. As shown in an earlier section, the substitution of beta-hydroxyl for beta-chlorine in dimethyl-beta-chloroethylamine removed the ability of the compound to produce waltzing. It is of interest to note (table III, E), that despite the presence of the dimethyl group and the beta-chlorine, the waltzing effect is not elicited when a phenyl group is substituted for one of the beta hydrogens in the chlorinated chain of dimethyl-beta-chloroethylamine. The resulting compound dimethyl-beta-phenyl-beta-chloroethylamine is more toxic than dimethyl-beta chloroethylamine. Dialkyl-gamma-chloro-n-propylamines have not been tested. However, diethyl-gamma-trichloro-n-propylamine and diethyl-epsilon-trichloropentylamine do not produce the waltzing effect.

7. None of a series of bis(beta-chloroethyl) amines described by Goldin, et al. and Landing, et al. (1, 2) produced the waltzing syndrome. Neither did tris (beta-chloroethyl) amine, nor did the various tetrakis-beta-chloroethylamines produce the waltzing syndrome. In general, these compounds are considerably more toxic than the waltzer producing compounds (1, 2). In this connection it is of interest to note that Anslow, et al. (11) reported that rats exposed to HN1 and HN2 vapor, and to intravenous administration of these agents occasionally exhibited neurologic injury on the third or fourth day after treatment. The

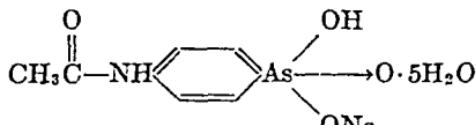
symptoms included increased irritability, abnormal posture and movement, "progressing in the severe cases to apparently severe involvement of the vestibular and cochlear mechanisms. Usually death rapidly followed the onset of these extreme effects, but among survivors with less severe injury hyperirritability, persisting for weeks, remained the only sign of injury." Thus, there is apparently some similarity of effect of the bis, tris, and tetrakis compounds to the waltzing effect. However, our observation has been that the mono-beta-chloroethylamines were the only mustards in which the effects persisted indefinitely.

Of particular interest is the compound ethyl-beta-chloroethyl-gamma-chloro-n-propylamine listed in table III, F. This compound bears a structural resemblance to both the mono-beta-chloroethylamines and the bis-(beta-chloroethyl) amines. Mice injected with this compound at concentrations in the area of the LD<sub>50</sub> showed all of the symptoms of the waltzing syndrome twelve hours after injection. However, the effects wore off in survivors after ninety-six hours. The compound is more than twice as toxic as ethyl-n-propyl-beta-chloroethylamine.

Two additional compounds of this type, listed in table III, F, namely,<sup>4</sup> N,N'-(beta-chloroethyl)-1,4-piperazine dihydrochloride, and N,N'-ethyl-N,N'-(beta-chloroethyl) ethylenediamine dihydrochloride are likewise of interest. The compounds structurally resemble the bis (beta-chloroethyl) amines in that they each have two beta-chloroethyl groups. However, the beta-chloroethyl groups are on separate nitrogen atoms so that the compounds also have a structural similarity to the waltzer-producing compounds. Neither of these compounds produced the waltzing syndrome, though some hyperactivity appeared in non-survivors of mice injected with N,N'-(beta-chloroethyl)-1,4-piperazine dihydrochloride. The order of magnitude of the toxicity of the two compounds indicates resemblance to the bis(beta-chloroethyl)amines.

8. Philips and Gilman (10) have shown that quaternary compounds containing N-beta-chloroethyl groups are relatively non-toxic and have little effect on the hematopoietic system. The compounds they cite are bis-(beta-chloroethyl) compounds. That a quaternary compound similar in structure to the effective dimethyl-beta-chloroethylamine does not produce the waltzing effect is shown in table III, G. Also included in the table is choline chloride which has a negative waltzing effect.

9. The occurrence of central nervous system lesions after exposure of mice to the various agents studied is indicated in table IV.<sup>5</sup> Also tested were two arsenicals, one of which arsacetin



<sup>4</sup> These compounds were obtained from Merck & Co., Inc., Rahway, N. J.

<sup>5</sup> The authors wish to thank Dr. Stanley H. Durlacher for consultation in examination of the sections.

TABLE III  
Compounds which do not produce the waltzing syndrome

CHEMICAL NAME	FORMULA	LD <sub>50</sub> mM/kgm.
A. Substitution of hydroxyl for β-chlorine		
Dimethyl-β-hydroxyethylamine		2.620
Diethyl-β-hydroxyethylamine		1.640
β-hydroxyethyl-piperidine-HCl		2.720
β-hydroxyethyl-morpholine-HCl		23.600
B. Primary and secondary amines		
β-chloroethylamine-HCl		19.000
Methyl-β-chloroethylamine-HCl		17.700
Ethyl-β-chloroethylamine-HCl		7.790
Propyl-β-chloroethylamine-HCl		2.550

TABLE III—Continued

CHEMICAL NAME	FORMULA	LD <sub>50</sub>
C. Substituents on the alkyl carbons of dimethyl-beta-chloroethylamine		
Dibenzyl-β-chloroethylamine·HCl		0.503 mM/km.
Di(p-chlorobenzyl)-β-chloroethylamine·HCl		ca. 1.640 ca. 1.640
Di(p-methoxy-benzyl)-β-chloroethylamine·HCl		ca. 1.750 ca. 1.750
D. Substitution of beta-bromine for beta-chlorine		
Dimethyl-β-bromoethylamine·HBr		0.210 0.210
Phenyl-β-bromoethylamine·HBr		1.210 1.210
E. Other dialkyl amines		
Dimethyl-β-phenyl-β-chloroethylamine·HCl		0.091 0.091

TABLE III—Continued

CHEMICAL NAME	FORMULA	LD <sub>50</sub> mM/kgm.
E. Other dialkyl amines		
Diethyl- $\gamma$ -trichloro-n-propylamine·HCl	$\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{N}-\text{CH}_2\text{CH}_2-\text{C}(\text{Cl})-\text{Cl} \cdot \text{HCl} \\   \\ \text{C}_2\text{H}_5 \end{array}$	0.667
Diethyl- $\epsilon$ -trichloropentylamine·HCl	$\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{N}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(\text{Cl})-\text{Cl} \cdot \text{HCl} \\   \\ \text{C}_2\text{H}_5 \end{array}$	1.410
F. Other related beta-chloroethylamines		
* Ethyl- $\beta$ -chloroethyl- $\gamma$ -chloro-n-propylamine·HCl	$\begin{array}{c} \text{CH}_3\text{CH}_2 \\   \\ \text{N}-\text{CH}_2\text{CH}_2\text{Cl} \cdot \text{HCl} \\   \\ \text{ClCH}_2\text{CH}_2\text{CH}_2 \end{array}$	0.112
* N-N'-( $\beta$ -chloroethyl)-1,4-piperazine·2HCl	$\begin{array}{c} \text{N}-\text{CH}_2\text{CH}_2\text{Cl} \\   \\ \text{N} \text{---} \text{C}_6\text{H}_4 \text{---} \text{N} \\   \\ \text{N}-\text{CH}_2\text{CH}_2\text{Cl} \\ -2\text{HCl} \end{array}$	0.021
N-N'-ethyl-N,N'-( $\beta$ -chloroethyl)-ethylenediamine·2HCl	$\begin{array}{c} \text{C}_2\text{H}_5-\text{N}-\text{CH}_2\text{CH}_2\text{Cl} \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_2\text{H}_5-\text{N}-\text{CH}_2\text{CH}_2\text{Cl} \\ -2\text{HCl} \end{array}$	0.010
G. Quaternary ammonium compounds		
$\beta$ -chloroethyl-trimethylammonium chloride	$\begin{array}{c} \text{CH}_3 \\   \\ \text{CH}_3-\overset{+}{\text{N}}-\text{CH}_2\text{CH}_2\text{Cl} \quad \text{Cl}^- \\   \\ \text{CH}_3 \end{array}$	0.410
Choline chloride	$\begin{array}{c} \text{CH}_3 \\   \\ \text{CH}_3-\overset{+}{\text{N}}-\text{CH}_2\text{CH}_2\text{OH} \quad \text{Cl}^- \\   \\ \text{CH}_3 \end{array}$	2.630

\* Produced transient waltzing.

was reported by Ehrlich (5) and Morgan (12) to produce waltzing after administration to mice. All four mustards marked as producing lesions gave cerebellar damage, and three of the four produced brainstem lesions, most marked in the medulla. No central nervous system lesions could be detected, by the techniques employed, in animals treated with arsacetin. Inner ear or auditory nerve damage was not observed at any time after treatment with any of these compounds, by the methods used.

TABLE IV

*The occurrence of central nervous system lesions after exposure of mice to waltzer-inducing and related compounds*

CHEMICAL NAME	LD <sub>50</sub>	DOSE	WALTZING SYMPTOMS	LESIONS
	mM/kgm.	mM/kgm.		
Dimethyl-β-chloroethylamine·HCl	1.950	2.090	+	+
Dimethyl-β-hydroxy-ethylamine·HCl	2.620	0.400	-	-
Diethyl-β-chloroethylamine·HCl	0.414	0.726	+	+
Diethyl-β-hydroxy-ethylamine·HCl	1.640	1.070	-	-
Ethyl-β-chloroethylamine·HCl	7.790	7.640	-	-
Methyl-ethyl-β-chloroethylamine·HCl	1.300	2.490	+	++
n-Propyl-β-chloro-ethylamine·HCl	2.550	0.796	-	-
β-chloroethyl-morpholine·HCl	0.865	1.080 0.810 0.540	+	++ ++ ++
β-hydroxyethyl-morpholine·HCl	23.600	14.900	-	-
Arsacetin	ca 5.390	6.740 3.370	+	-
Atoxyl	1.000	3.780 2.830	-	-

*Cerebellar lesions.* During the first twenty-four hours after the injection of a waltzer-inducing mustard, eosinophilia and shrinkage of occasional Purkinje cells were seen. No necrosis of these cells was found, nor was reactive gliosis in the Purkinje cell layer noted. This change appeared to represent a nonspecific toxic effect of these compounds, and, because of its transience and lack of sequelae, appeared to play no role in the causation of the permanent neurologic symptoms described above.

From two to five days after injection of these compounds, pyknosis and fragmentation of the cells of the granular layer of the lingula of the anterior lobe of

the cerebellum were found. This lesion varied in severity with the different effective compounds, being more marked after beta-chloroethylmorpholine and methyl-ethyl-beta-chloroethylamine than after the other two. The lesion also varied somewhat in extent, since minor degrees of this process could be seen in the folia of the anterior lobe adjoining the lingula after administration of beta-chloroethylmorpholine. Reactive gliosis was negligible in these lesions and, in animals killed more than ten days after exposure, the presence of any abnormality was difficult to detect, presumably because of condensation of the stroma of the granular layer to produce a cell density approximating the normal.

*Brain stem lesions.* With three of the four effective mustards studied (beta-chloroethyl-morpholine, diethyl-beta-chloroethylamine, and dimethyl-beta-chloroethylamine), in animals killed later than three days after injection, scattered small areas of gliosis were found in the brain stem, medulla, and upper cord. These were most numerous after the dimethyl-beta-chloroethylamine, and in one animal treated with this agent, serial sections of the brain stem, medulla, and cord showed approximately thirty of these lesions, involving the restiform body, the vestibular nuclei, and the tectospinal, descending spino-cerebellar, and lateral pyramidal tracts. These lesions differ from those produced in the brains of rabbits by intravenous methyl-bis (beta-chloroethyl) amine (13), being smaller, more sharply localized, and less acute in appearance.

**DISCUSSION.** The characteristic properties of the nitrogen mustards, including their high reactivity with cellular constituents, are traceable to the formation in polar solvents of the highly reactive ethylenimonium ion. The schema of hydrolysis has been described by Bergmann, et al. (7, 8, 9), Gilman and Philips (6) and Philips and Gilman (10).

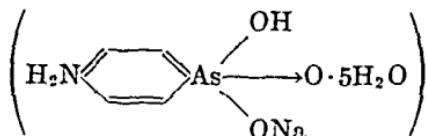
There is some indication that the action of the bis compounds includes biological action which could elicit the waltzing syndrome, but that this action is masked by other toxic characteristics of the compounds, which result in death at concentrations required for the production of the waltzing effect. This is supported by the following observations: (a) The bis-beta-chloro compounds, as indicated above, are of a higher order of toxicity than the mono-beta-chloro compounds. (b) Mice which receive supra-lethal doses of the bis compounds exhibit hyperactivity and neurological behavior resembling in some degree the waltzing syndrome. Since these mice die one cannot test to what extent this portion of their behavior might have persisted, had the mice lived. (c) Median lethal dose survivors of bis compounds may exhibit some hyperactivity during initial stages after administration of the agents. In our experience this hyperactivity does not persist. However, Anslow et al. (11) have found instances in which such hyperactivity did persist, after exposure of rats to vapors of HN1 and HN2 and after intravenous administration of these agents. (d) Dimethyl-beta-bromoethylamine, though chemically similar to the beta-chloroethylamines in that it presumably is subject to imonium ion formation does not produce the waltzing syndrome. Its toxicity is of a considerably higher order than that of its corresponding chlorinated compound, dimethyl-beta-chloro-ethylamine, though not as toxic as typical bis compounds. The higher toxicity may mask the waltz-

ing effect by not permitting survival at higher doses of the compound. (e) The compounds N,N'-(beta-chloroethyl)-1,4-piperazine dihydrochloride and ethyl-beta-chloroethyl-gamma-chloro-propylamine produced transient hyperactivity and waltzing. The compounds have structural similarity to both the mono and bis compounds, and both are more toxic than the mono compounds. Again, had there been survivors at higher doses, the waltzing effect might have been elicited.

None of these arguments are more than suggestive and certainly not conclusive. The possibility cannot be ruled out that the mono-beta-chloro compounds are entirely qualitatively different, biologically, from the bis-beta-chloro compounds, though the data do not seem to support this hypothesis.

Apparently only chlorinated amines in a fairly circumscribed range of structure are capable of eliciting the waltzing syndrome. The beta-chloroethylamine structure is common to them all, and is apparently one of the prerequisites for the production of the effect. A dialkyl structure or heterocyclic ring, as in beta-chloroethyl-piperidine, is apparently also necessary.

Ehrlich (cited by Gruneberg (5)) and Morgan (12) used arsacetin and described an effect in mice similar to that which we have obtained with the waltzer producing mustards. We have verified their observations. However, in Carworth mice we did not get the extent of waltzing nor did the effect appear with as equal regularity as it does with the waltzer producing mustards. Atoxyl pro-



duced hyperactivity but no waltzing. There does not appear to be any correlation in structure between these arsenicals and the mustards.

The question arises as to whether the behavior pattern of the mice which were injected with the various compounds which produced waltzing was similar for every compound, in every respect. We were not able to observe any gross differences in the behavior of the waltzing mice for the various compounds. That behavior differences in detail may exist can be ruled out only after extensive behavior studies.

Either of the lesions formed in waltzer-induced mice, namely, cerebellar or axial, seems adequate to account for the symptoms displayed by mice treated with adequate doses of these agents. The lingula of the anterior lobe of the cerebellum is, at least in higher mammals, the recipient of impulses from the lateral vestibular nuclei, carried by the juxtarestiform body of the inferior cerebellar peduncle, and shares in the vermis' functions of coordination of the axial musculature with optic and visual stimuli, and of control of bilateral synergic motions.

Scattered lesions of the medulla and upper cord lead, by disturbance of the functions of the tracts mentioned above, among others, to tremor, incoordination, weakness and/or spasticity of muscles, and to sensory disturbances (cf.

for example the symptoms of multiple sclerosis). As mentioned above, either of these processes can probably account for the neurologic manifestations described above, and the exact roles played by the two in the production of these disturbances cannot be decided from the pathologic data obtained to date.

#### SUMMARY

1. A series of nitrogen mustard analogues were studied and the essential structural groupings necessary to produce the neurological syndrome of waltzing in mice were identified.
2. The compounds which produced the waltzing syndrome were dialkyl and heterocyclic-beta-chloroethylamines.
3. No waltzing was elicited when the following structural modifications were made:
  - a) Substitution of a hydroxyl group for the beta chlorine.
  - b) Substitution of a beta-phenyl group for one of the beta hydrogens in the chlorinated chain.
  - c) Substitution of bromine for the beta chlorine.
  - d) Introduction of phenyl groups into the dialkyl carbons.
4. Primary and secondary beta-chloroethylamines; related quaternary compounds; and bis, tris, and tetrakis beta-chloroethylamines did not elicit the effect.
5. Two compounds similar in structure to both the mono-beta-chloroethyl amines and bis(beta-chloroethyl)amines produced transient waltzing.
6. Cerebellar and axial lesions found in induced waltzers could account for the behavior pattern of the mice.
7. The chemical relationships of these compounds and their mechanism of action are discussed.

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# CONCERNING THE INABILITY OF 2,3 DITHIOPROPANOL<sup>1</sup> (BAL) TO PREVENT OR TO MODIFY THE ACUTE RENAL INJURY INDUCED BY URANIUM NITRATE\*

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2,3 Dithiopropanol (BAL) is one of a large number of chemically closely related bodies developed by Peters, Stocken and Thompson (1) in an attempt to find a substance which would so influence tissues as to modify the toxic action of gaseous substances containing arsenic. The efficacy of certain of the dithiols in this connection represents one of the major contributions of medical research during the recent war. Following this discovery numerous investigations have been undertaken of this action within various tissues susceptible to the toxic action of the salts of the heavy metals, the rate and form of excretion by the kidney of such salts and finally, the clinical application of such information in the treatment of poisoning by such bodies.<sup>2</sup>

The present investigation was undertaken as a result of the observations made by Gilman, et al. (2) and by Longcope and Leutscher (3) on the treatment in animals and in man of acute mercury poisoning by the use of BAL. The acute injury to the kidney by bichloride of mercury is very largely confined to the epithelium of the proximal convolution of the renal nephron. Other segments of this structure are implicated in the toxic effect to a far less extent and furthermore damage to them occurs late. The specialized vascular structures of the kidney, the glomeruli, are spared until still later in the renal injury (4). A very similar statement may be made for the nephrotoxic action of uranium nitrate (5, 6). In this intoxication the distal convolution of the renal nephron shows evidence of a delayed implication in the injury and although the glomeruli may appear histologically preserved during the acute phase of the intoxication other than an acute engorgement of the capillary tufts, the fact that an injury to these structures has been effected is shown by the inauguration of processes of repair of a chronic obliterative order (7, 8). The similarity in the dominant locus of toxic action of both bichloride of mercury and uranium nitrate in the kidney and the fact of the ability of a dithiol to prevent or to relieve such an injury from mercury has resulted in the present study of the efficacy of such a chemical body in preventing or in modifying the nephrotoxic action of uranium nitrate.

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<sup>1</sup> 2,3 Dithiopropanol (BAL), 10 per cent, benzyl benzoate 20 per cent in peanut oil.

<sup>2</sup> As a result of the theoretical and applied significance of these contributions The Journal of Pharmacology and Experimental Therapeutics, Vol. 87, no. 4, August, 1946, and The Journal Of Clinical Investigation, Vol. 25, no. 4, July, 1946, are largely devoted to presenting this literature to date.

In the investigation which is to follow, the dog was chosen for study on account of the years of experience with this animal under the influence of various amounts of uranium nitrate both during the period of acute renal injury and that of renal repair which finally lead to the development of a characteristic type of chronic nephropathy. The functional expression of such periods of injury are invariably of the same order though varying in their quantitative expression. They afford a functional constant depending upon the amount of the nephrotoxic agent employed and the age of the animal in which the reaction occurs (9).

Two types of control experiments have been necessary in this investigation. First, the use of a series of animals to demonstrate whether or not 15 mgm. of BAL per kgm. would in itself induce a renal injury as has recently been shown to develop in the liver from the use of 30 mgm. of dithiol in the normal dog (10) and, second, the demonstration by the use of 4 mgm. of uranium nitrate per kgm. in another series of control experiments that this substance would induce in a six-day period allocated for observation a renal injury dependably constant in its intensity as shown by both functional and anatomical studies. Both of these control groups of dogs as well as those used in the experiments proper were fed on Purina Dog Chow and table scraps. No restriction was placed on the water intake.

**CONTROL EXPERIMENTS WITH 2,3 DITHIOPROPANOL.** Four adult dogs were used in this group of experiments. The animals were given intramuscularly at twelve-hour intervals for four days 15 mgm. of the dithiol per kgm. Renal functional studies were made on the second and fourth day of this period and following the final observation biopsy material was obtained from the left kidney for histological study. The findings in Experiment 4 made at the termination of the dithiol injections are characteristic for the group. The urine both before and after the dithiol was free from albumin, glucose and diacetic acid. An occasional cylindroid was found in the urine. The carbon dioxide plasma tension was 48.3,<sup>3</sup> the elimination of phenolsulphonephthalein in a one-hour period was 64 per cent while the whole blood gave the following values in mgm. per 100 cc.: urea nitrogen 8, non-protein nitrogen 24.6 and creatinine 1.51. The histological study of biopsy material from the kidney gave no evidence of definite structural change. The glomeruli were normal in appearance without active engorgement of the capillaries, the cells of the tubular portion of the nephron were normal in their staining reactions and failed to show any pathological response such as albuminous degeneration or vacuolation which would lead to an increase in cell volume. There was no intertubular edema or proliferation of connective tissue cells. The conclusion is justified that the use of 15 mgm. per kgm. of BAL at twelve-hour intervals over a period of four days fails to induce a renal injury in the normal dog (fig. 1).

**CONTROL EXPERIMENTS WITH URANIUM NITRATE.** Four normal dogs were used in this series of experiments. The animals were given one subcutaneous injection of 4 mgm. of uranium nitrate per kgm. Studies of the blood and urine

<sup>3</sup> Carbon dioxide plasma tension is expressed as mm. Hg

were made on the third and sixth days of the intoxication. At the time of the last observation biopsy material was obtained from the left kidney. Only the data obtained on the sixth day of the experiments will be presented. It was at this period in the experiments which are to follow that a dithiol was used in these animals acutely nephropathic from uranium.

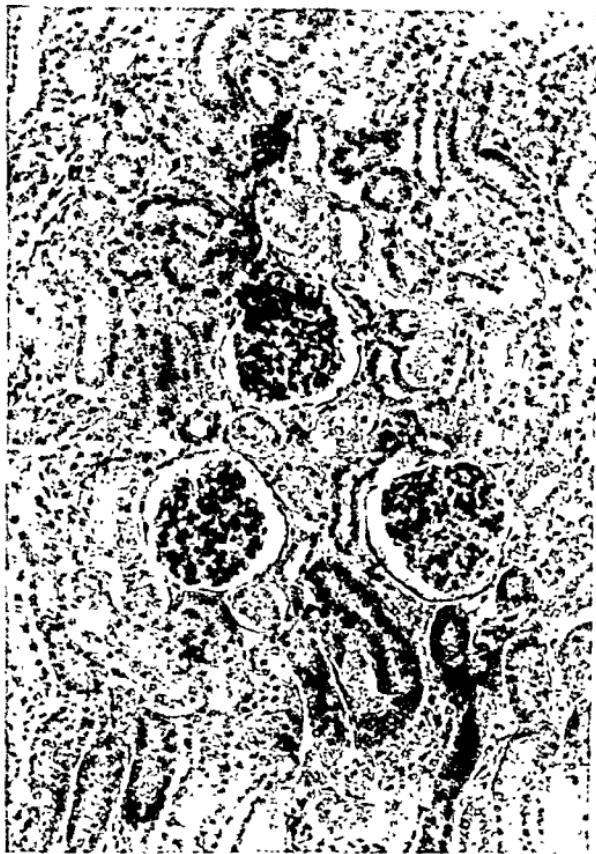


FIG. 1. MICROPHOTOGRAPH. ZEISS X 235, HEMATOXYLIN AND EOSIN

The figure is from biopsy material of the kidney of the animal of Exp. 4 in that group of our intervals at capillaries. The tubule shows no evidence of a dithiol

The response of the animal of Experiment 2 in this series of control animals is characteristic for the group. The preliminary studies of the urine of this dog showed it to be normal. Casts were not present. The carbon dioxide plasma tension was 48.7. There was an elimination of 62 per cent of phenolsulphone-phthalein in a first hour period. The whole blood gave the following values

in mgm. per 100 cc.: urea nitrogen 7.4, non-protein nitrogen 26.7 and creatinine 1.21. On the sixth day of the uranium nitrate intoxication the carbon dioxide plasma tension had decreased to 30.1. The output of phenolsulphonephthalein was 5 per cent. The urine contained a heavy precipitate of albumin with numerous finely and coarsely granular casts. Urea nitrogen had increased to a retention value of 23.8 mgm., non-protein nitrogen to 82.4 mgm. and creatinine to 3.2 mgm. per 100 cc. of blood. The histological study of renal tissue obtained on this sixth day of the intoxication showed the glomerular capillaries to be engorged with blood, distending the capsule and increasing the volume of the glomerular bodies. There was no subcapsular glomerular hemorrhage or the appearance of thrombi in the glomerular capillaries. Not infrequently a small amount of poorly stained granular detritus could be demonstrated between the capillary tufts and Bowman's capsule. The tubular portion of the nephron showed the characteristic election of the epithelium of the proximal segment of the nephron for the dominant action of uranium. These cells were acutely swollen, usually from an accumulation of granular material of assumed protein derivation and more rarely by the appearance of a vacuolar type of degeneration. Complete necrosis of such cells was rarely observed. The nuclei in general stained well and were not fragmented. The epithelium of the distal convoluted segment of the tubule has shown evidence of injury of the same pathological order but to a far less extent than that which occurs in the proximal convoluted segment. The cells of the descending and ascending limb of Henle's loop are spared in the uranium intoxication. The conclusion is permissible that uranium nitrate when given subcutaneously in the amount of 4 mgm. per kgm. induces by the sixth day of the intoxication an injury to the kidney which in terms of actual degeneration is largely localized in the cells of the proximal convoluted segment of the renal nephron. Associated with this dominant injury, with less evidence of injury to other portions of the epithelial structure of the nephron and with an acute engorgement of the glomerular vessels, the animals developed a marked albuminuria with casts, a glycosuria and an associated appearance of diacetic acid in the urine. There occurs a decrease in carbon dioxide plasma tension, a rapid reduction in the elimination of phenolsulphonephthalein and a retention of urea nitrogen, non-protein nitrogen and creatinine (fig. 2).

THE PREVENTIVE AND REMEDIAL INFLUENCE OF 2,3 DITHIOPROPANOL IN ACUTE URANIUM NITRATE INTOXICATIONS. Fifteen dogs have been intoxicated by one subcutaneous injection of 4 mgm. of uranium nitrate per kgm. Depending upon the time at which these animals were given a dithiol in relationship to the commencement of the uranium intoxication they may be divided into three groups.<sup>4</sup>

*The animals of group I.* Five animals of this group were given intramuscularly 15 mgm. of BAL per kgm. at twelve-hour intervals for two days prior to the use of the uranium salt. They serve as a group to demonstrate any prophylactic value the dithiol might have in terms of its ability to modify or prevent the development of a renal injury from uranium. At the expiration of this period allocated for the use of the dithiol the dogs were given one subcutaneous injection

<sup>4</sup> Uranium nitrate was given immediately following the last dose of dithiopropanol.

of 4 mgm. of uranium nitrate per kgm. The course of Experiment 3, Group I, is characteristic for the group as a whole. A preliminary period of study showed the animal to be normal as indicated by the findings in the blood and urine.

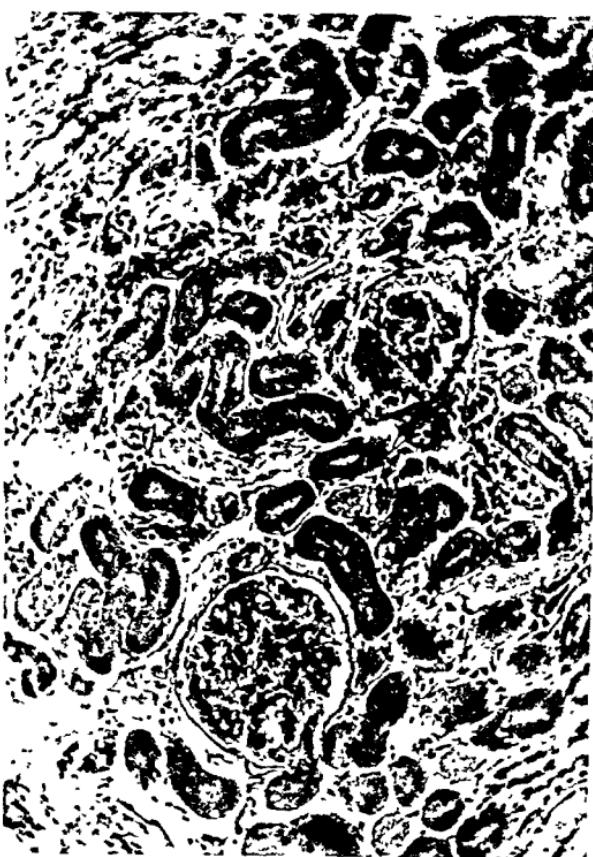


FIG. 2. MICROPHOTOGRAPH. ZEISS X 235, HEMATOXYLIN AND EOSIN

The figure is from biopsy material of the kidney of the animal of Exp. 2 in that group of control animals which were given one subcutaneous injection of 4 mgm of uranium nitrate per kgm. No dithiol was used in this series of control experiments. The biopsy was made on the sixth day of the uranium nitrate intoxication. The figure shows the characteristic and dominant location in the action of uranium to take place in the proximal convoluted segment of the renal nephron. The epithelium in this location is edematous, the cells in general closing the tubular lumen. In certain of the tubules these changes have advanced to a state of epithelial necrosis with a loss of cell nuclei or a failure of those remaining to stain. The two glomeruli appearing in the figure are engorged with blood and in the smaller of these structures a subcapsular exudate has partially compressed the glomerular tufts. An intertubular exudate is to be observed in the areas adjacent to the glomeruli.

On the terminal day of the use of the dithiol no significant change from the normal had occurred in the blood and urine. The urine did not contain albumin, glucose or diacetic acid. The carbon dioxide plasma tension was 48.9. There was an elimination of 63 per cent of phenolsulphonephthalein in an hour period.

The whole blood gave the following values in mgm. per 100 cc.: urea nitrogen 12.2, non-protein nitrogen 30.1 and creatinine 1.71. On the third day following the commencement of the uranium nitrate intoxication the urine contained a heavy precipitate of albumin and both glucose and diacetic acid. Numerous finely and coarsely granular casts and occasional fatty casts were present. The carbon dioxide plasma tension was reduced to 35.2. There was only an output of 12 per cent of phenolsulphonephthalein. Blood urea nitrogen was increased to 72.4 mgm., non-protein nitrogen to 133.2 mgm. and creatinine to 3.7 mgm. per 100 cc. of blood. On the sixth day of the intoxication other than the reduction in volume output no significant change had developed in the urine. There was no elimination of phenolsulphonephthalein. Carbon dioxide plasma tension was further reduced to 23.8. Blood urea nitrogen had increased to 105.5 mgm., non-protein nitrogen to 187.5 mgm. and creatinine to 6.28 mgm. per 100 cc. of blood. The animal gave clinical evidence of a profound state of intoxication. The experiment was terminated. The histological study of the kidney showed the usual predominant injury to the epithelium of the renal nephron with the most advanced changes in this tissue localized in the proximal convoluted segment. Here the cells in many instances were completely necrotic with a disappearance of nuclear material. In other tubules the most marked change was that of a severe edema, partially or completely closing the tubular lumen. In such cells the nuclei were small but not fragmented, stained intensely and were usually found near the basement membrane of the tubule. There was no inter-tubular exudate. The glomeruli were engorged with blood, usually obliterating the subcapsular space or in instances in which such a change had not occurred there was to be found in this space a zone of amorphous, granular material. In no instance were the glomeruli in a state of compression from such an accumulation (fig. 3). From the above account of the course of events developing in the animals of Group I that first received BAL before the commencement of the uranium intoxication the deduction is made that the dithiol in the amount administered to the animals not only had no effect in preventing the uranium nitrate intoxication but that it increased the severity of the uranium injury as indicated by both the changes in the blood and urine and by the extent and severity of the pathological reaction on the part of the kidney. A similar observation has recently been made for the liver following the use of 30 mgm. of dithiol per kgm. (11).

*The animals of group II.* The five animals of this group, after a period allocated for normal observations, were given one subcutaneous injection of 4 mgm. of uranium nitrate per kgm. Immediately following such an administration, the dogs were given intramuscularly 15 mgm. per kgm. of BAL and these injections were repeated at twelve-hour intervals for two days. The animal of Experiment 2 has shown a response which is characteristic for the members of the group. The dog had a normal urine. The elimination of phenolsulphonephthalein in an hour period was 68 per cent. The plasma carbon dioxide tension was 44.7. The blood showed a urea nitrogen of 13.5, non-protein nitrogen 29.4 and creatinine 1.32 mgm. per 100 cc. On the third day of the uranium ni-

trate intoxication during the first forty-eight hours of which the dithiol had been given at twelve-hour intervals the urine contained a large amount of albumin, numerous coarsely granular casts, glucose and diacetic acid. The plasma carbon dioxide tension had been reduced to 30.5. There was no measureable elimina-



FIG. 3. MICROPHOTOGRAPH. ZEISS X 235, HEMATOXYLIN AND EOSIN.

The figure is from the kidney of the animal of Exp. 3, Group I, of those animals which were first given at twelve-hour intervals for two days 15 mgm. of BAL and then intoxicated by one injection of 4 mgm. of uranium nitrate per kgm. The figure shows in general a complete necrosis of the epithelium of the proximal convoluted tubules. This terminal change in other tubules of this segment of the nephron is preceded by a marked edema of the cells and by either the disappearance or imperfect staining of the nuclei. Two glomeruli are shown in the figure. The larger of the two is engorged with blood which increases its size. The smaller of the two structures is in the early stages of capillary compression by an exudate. The use of a dithiol prior to an intoxication by uranium intensifies the nephrotoxic action of this substance.

tion of phenolsulphonephthalein. Urea nitrogen had increased to 26.9, non-protein nitrogen to 47.2 and creatinine to 2.42 mgm. per 100 cc. of blood. On the sixth day of the intoxication the plasma carbon dioxide tension had been further reduced to 25.0. There was no elimination of phenolsulphonephthalein. Urea

nitrogen had increased to 77.8, non-protein nitrogen to 133.4 and creatinine to 5.8 mgm. per 100 cc. of blood. The experiment was terminated. The kidney showed a pathological response of the same type but of a more severe order than that of the animals in Group I that received the dithiol prior to the uranium intoxication but not following its development as was the case with the group of animals under consideration, Group II. The necrosis of the epithelium of the proximal convoluted segment of the nephron is uniform and complete and in other epithelial segments there is marked evidence of injury. It would appear from a study of the kidney from both of these groups of animals, Groups I and II, that the use of a dithiol not only leads to an intensification of the uranium action at its point of election, the proximal segment of the renal nephron, but that it enables this action of epithelial degeneration to extend to other segments of the nephron which in the absence of a dithiol influence are either not affected or injured to a very slight extent. The glomeruli are histologically preserved. The glomerular capillaries are engorged with blood and show a nuclear increase. Amorphous material is rarely seen in the subcapsular space. There is no inter-tubular exudate (fig. 4).

A study of both the functional and anatomical changes developing as a result of the renal injury in the animals of Group II, permit the conclusion that the use of a dithiol at the time of the establishment of a uranium intoxication and continued into this period for forty-eight hours had not only no influence in protecting the kidney against the toxic action of this substance but that its use causes both an intensification of and an epithelial extension in the toxic action of uranium.

*The animals of group III.* The five dogs of this group, following the period allowed for normal observations, were given 15 mgm. per kgm. of BAL intramuscularly for two days at twelve-hour intervals, then given one subcutaneous injection of 4 mgm. of uranium nitrate per kgm. to be followed by a continuation of the dithiol injections for a second two-day period. The animal of Experiment 2 shows a response both functionally and anatomically which is characteristic for the group. The urine of the animal was normal. The elimination of phenolsulphonephthalein was 51.8 per cent in an hour period. The plasma carbon dioxide tension was 48.7. The blood urea nitrogen was 13.6, non-protein nitrogen 17.3 and creatinine 1.32 mgm. per 100 cc. At the end of the second day of the dithiol injections there was no change of significance in the blood or urine. The urine showed a trace of albumin with an occasional hyaline cast. There was no detectable change in the plasma binding power for carbon dioxide. The elimination of phenolsulphonephthalein was not reduced below its normal percentage of elimination. There was no nitrogen or creatinine retention. On the third day of the experiment following the commencement of the intoxication by uranium nitrate, during two days of which period the dithiol had been continued, the plasma combining power for carbon dioxide was reduced to 30.2. The elimination of phenolsulphonephthalein was reduced to an undeterminable trace. The blood showed an increase in urea nitrogen to 67.6, non-protein nitrogen to 100.0 and creatinine to 4.81 mgm. per 100 cc. The urine contained a

heavy precipitate of albumin and very numerous coarsely granular and fatty casts. On the sixth day of the experiment the plasma carbon dioxide tension was 20.1. The animal was comatose with an air-hunger type of breathing.



FIG. 4. MICROPHOTOGRAPH. ZEISS X 235, HEMATOXYLIN AND EOSIN

The figure is from the kidney of the animal of Exp. 2, Group II, of those animals which were first given 4 mgm. of uranium nitrate per kgm. to be immediately followed by the use of 15 mgm. of the dithiol at twelve-hour intervals for two days. The figure shows the same order and intensity of injury to the kidney as has been described for the kidney of the animal of Exp. 2, Group I, in which group the dithiol was given before the commencement of the

The figure from the kidney of the animal under discussion (Exp. 2, complete necrosis of the epithelium of the proximal convoluted tubule) appears in a state of acute edema not infrequently of a vacuolar type.

Two glomeruli are shown which vary in size and degree of engorgement. In both structures there is a commencing subcapsular exudate. The use of a dithiol at the time of the commencement of and during the course of a uranium intoxication intensifies the nephrotoxic action of uranium.

No urine was available for the determination of phenolsulphonephthalein. The retention of urea nitrogen had increased to 81.6, non-protein nitrogen to 200+ and creatinine to 6.84 mgm. per 100 cc. of blood. The animal died three hours after these determinations were made and was at once autopsied. Histologically

the kidneys showed the same order of selective epithelial injury which has been described for all of the animals in the experiments that received uranium nitrate. In the animals of this group, Group III, as was the case with those comprising Groups I and II that were also subjected to the action of a dithiol in addition to the uranium effect, the selective action of the uranium in the proximal convoluted tubules is more severe and is associated with an extension of the epithelial injury to other segments of the nephron. In addition to these changes in the epithelium there develops in the kidneys of the animals of Group III a superimposed vascular injury. This is shown in the glomeruli by a great increase in the size of certain of these structures due to an engorgement and over-distention of the capillaries with blood. In such structures a variable amount of granular material collects between Bowman's membrane and the capillary tufts. In other glomeruli, and this is the characteristic change, the tufts of capillaries may have entirely disappeared, the space surrounded by the capsule being filled with blood in various stages of disintegration. Numerous glomeruli show the capillary tufts at different stages of their compression by a coagulated exudate usually containing both preserved and fragmented nuclei. In addition to the vascular glomerular injury, the intertubular vessels are greatly distended with blood, which increase has ruptured the vessels appearing free in the kidney substance.

From the data acquired by the study of the animals of Group III, the conclusion is permissible that the use of 15 mgm. BAL per kgm. when given intramuscularly for two days at twelve-hour intervals both before and following the intoxication of the animals by one subcutaneous injection of 4 mgm. of uranium nitrate per kgm. not only fails to afford any protection against the renal injury from uranium but that it intensifies the action of this nephrotoxic substance. Such a renal injury is not only shown by the most severe and extensive tubular epithelial degenerative effect that occurs in the three groups of experimental animals but in addition the vascular tissue of the kidney becomes implicated in the process of degeneration.

**DISCUSSION.** The intracellular mechanism involved in the toxic action of uranium nitrate is not known. With certain limitations as to the amount of this substance which is employed to induce such a toxic expression the injury to the kidney is very largely confined to the epithelial tissue of the nephron and, like the reaction of the kidney to bichloride of mercury, to the proximal convoluted segment of the nephron. The accepted theory employed to explain the striking ability of a dithiol both to prevent and ameliorate such tissue degeneration from bichloride of mercury as well as from arsenic and salts of other of the heavy metals is that such bodies exert their toxic influence by inhibiting the effective action of certain intracellular enzyme systems so that intracellular respiration is either so modified or arrested that processes of cell degeneration supervene (12, 13, 14). Such an action is hypothesized to be brought about by the salt of the heavy metal combining with sulphydryl groups of intracellular enzymes. The use of a dithiol in such a circumstance, by furnishing free sulfhydryl radicals would enable the salt to become bound, prevent its continued action within the cell and facilitate its elimination by the kidney. In addition,

evidence is available to suggest that the use of a dithiol with its available sulfhydryl groups may enable such a body to compete effectively with intracellular enzymes for their bound toxic agents of this chemical order. Accepting the validity of this explanation it would appear that uranium in the form of the nitrate either fails to induce its nephrotoxic action by binding sulfhydryl groups essential for cellular respiration or that such a union is of such a loose nature that these atom groups are released during the elimination of uranium by the kidney permitting it to exercise its toxic action by a mechanism which at present is unknown (15, 16).

#### CONCLUSIONS

1. 2,3 Dithiopropanol in the amount used in these experiments (15 mgm. per kgm.) is nontoxic for the kidney in the sense that its use leads to no histologically demonstrable cell changes.
2. Uranium nitrate when given subcutaneously in the amount of 4 mgm. per kgm. induces a constant type of acute renal injury which is largely confined to the proximal convoluted segment of the renal nephron. The intensity of this action is to an extent determined by the age of the animal.
3. The use of 15 mgm. of 2,3 dithiopropanol given at twelve-hour intervals prior to the use of uranium nitrate has no effect as a prophylactic agent in preventing the renal injury from uranium. The severity of the injury is increased as is shown by the changes in the kidney and by the functional expression of these changes. Gilman and his associates (17) have made a rather similar observation in their studies of the influence of dithiols in cadmium poisoning, to the effect that it "enhances the toxicity of the metal for the kidney."
4. When an acute renal injury has been established by the use of one subcutaneous administration of 4 mgm. uranium nitrate per kgm., the subsequent employment of 15 mgm. per kgm. of 2,3 dithiopropanol at twelve-hour intervals for two days has no effect in decreasing the severity of the renal injury. The renal damage becomes intensified and renal function shows a correlation with these changes.
5. Finally, when 15 mgm. of 2,3 dithiopropanol are given to an animal at twelve-hour intervals for two days, then intoxicated with 4 mgm. of uranium nitrate and followed by a continuation of the dithiol at twelve-hour intervals for a second period of two days the toxic action of uranium manifests its greatest degree of renal toxic action. This action is shown by a more diffuse implication of the various segments of the renal nephron in the injury and by the ability of the uranium either acting as such or in conjunction with the dithiol to injure severely the vascular tissue of the kidney both in the glomeruli and in the intertubular vessels of the kidney.
6. The use of 2,3 dithiopropanol in a uranium nitrate intoxication even though it fails to show when used alone (see control experiments) either structural or functional renal changes, appears to so influence the epithelial tissue of the kidney as a sensitizing agent for uranium that the nephrotoxic action of this substance becomes more severe at its point of selective action in the proximal convoluted tubule and furthermore spreads to other segments of the renal nephron.

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# THE EFFECT OF CARDIAC GLYCOSIDES ON THE RESPIRATION OF CARDIAC MUSCLE<sup>1</sup>

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The effect of cardiac glycosides on oxygen utilization by the heart has attracted considerable attention for a number of years. In 1912, Rohde and Ogawa (1) found that a perfusion fluid containing 0.06 mgm. of strophanthin per 100 cc. of Ringer's solution increased oxygen consumption of isolated cat heart by about 20 per cent. Gottschalk (2) pointed out that this increase may have been due in part to the increase in heart rate which accompanied the addition of strophanthin. Employing the isolated frog ventricle, Gottschalk found that a concentration of 1:1,000,000, or less, of strophanthin caused negligible decreases in oxygen uptake, whereas concentrations ranging from 1:500,000 to 1:125,000 depressed oxygen consumption to values as low as 31 per cent of normal. Eismayer and Quincke (3) using the isolated frog ventricle found that strophanthin in a concentration of 1:10,000,000 increased oxygen consumption about 30 to 50 per cent, whereas concentrations of 1:333,000 to 1:72,000 decreased it about 30 per cent. In addition to this they found that the work done by the heart was decreased negligibly at the low concentrations of strophanthin, but markedly at the higher concentrations. David (4), using the frog's auricle, obtained essentially similar results, i.e., strophanthin in low concentrations caused a slight initial increase in oxygen uptake followed by a decrease which was accompanied by a decrease in amplitude and frequency of contraction. Higher concentrations produced a fall only in oxygen utilization accompanied by marked decreases in amplitude of contraction and frequency of the auricle. Victor (5) observed that ouabain increased the oxygen uptake of frog cardiac ventricular muscle in isotonic glucose. A concentration of 1:100,000 increased it an average of 13 per cent, 1:33,000 increased it 51 per cent, and a concentration of 1:10,000 an average of 76 per cent. The increase in oxygen uptake in Ringer's solution (0.65 per cent NaCl, 0.0075 per cent KCl, 0.01 per cent CaCl<sub>2</sub> and no buffer or substrate) was approximately 100 per cent when ouabain was present in a concentration of 1:33,000. Salomon and Riesser (6) reported that digitoxin in a concentration of 1:100,000 had no effect on the oxygen uptake of the isolated frog heart or minced frog, mouse or rabbit heart. Strophanthin in several concentrations was also reported to be without effect. Genuit and Haarmann (7) employed a brei prepared by cutting the cardiac tissue of rats or guinea pigs very finely with scissors. A concentration of strophanthin ranging from 1:3,000,000 to 1:100,000 exerted a slight but not clearly demonstrable accelerating effect on oxygen uptake of the tissue.

<sup>1</sup> This investigation was supported by a research grant from the United States Public Health Service.

It may be seen that up until about 1946 practically all experiments were conducted with either isolated whole heart, ventricle, auricle or with minced heart muscle. With regard to the isolated heart preparation, the possibility existed that the increased oxygen consumption in the presence of cardiac glycosides was due to an increase in the work of the heart. On the other hand, experiments with minced tissue showed little or no effect of the glycosides on the oxygen uptake. In this connection, the possibility arises that intact tissue is necessary for the demonstration of the accelerating effect of the glycosides.

In 1946, Lévy and her collaborators (8-12) published an extensive series of studies in which they observed the effect of several cardiac glycosides on mammalian heart slices. In general, they found that slices in the presence of dilute concentrations of glycosides showed an increased oxygen utilization, as compared with controls, during the first hour of respiration. However, during the second hour there was a smaller increase or even a decrease. In the presence of higher concentrations of glycosides there was a decrease in the oxygen uptake. Using a slightly different technique, in which ouabain was introduced during the course of the experiment, Wollenberger (13) obtained essentially similar results on guinea pig heart slices.

In conjunction with other studies in this laboratory (14, 15) on the action of scilliroside, it became of interest to study the effect of this glycoside on respiration of cardiac tissue.<sup>2</sup> The present paper is concerned with a study of the influence of the cardiac glycosides on the oxygen uptake of cat heart slices. The effect of varying concentrations of scilliroside and of glucose in the medium have been investigated. Because the results obtained have differed from those of Lévy and her collaborators (8-12) and of Wollenberger (13) with other glycosides, our studies have been extended to ouabain and digitoxin as well. Since it had been shown previously by Loewi (16), by Clark (17) and by others, that the action of the digitalis glycosides on the contraction of cardiac musculature was dependent on the calcium ion, it was of interest to determine the role which this ion played in the effect of the glycosides on respiration. In this connection, the effect of the magnesium ion and of the concentration of phosphate have also been studied.

**METHODS.** Respiration was measured by the Warburg manometric technique. Cats weighing 2.5 to 4.0 kgm. were anesthetized rapidly and lightly with ether and the hearts immediately removed to a pre-cooled Ringer-phosphate-glucose medium to be described below. All solutions in contact with the tissues were kept at about 5-10°C. until the vessels were placed in the 38°C.-water bath. The left ventricle was isolated, its epicardium removed and free-hand slices of approximately 0.8 mm. in thickness were cut and placed in a petri dish containing 10 cc. of the medium undergoing agitation by oxygenation. Slices selected for their thickness and uniformity, weighing approximately 100 or 150 mgm., were placed respectively in 1.8 cc. of medium in the micro-vessels or in 2.7 cc. of medium in the larger vessels. In a series of preliminary experiments it was found that slices thinner than those used in these experiments consumed about 50 per cent less oxygen. The side arm usually contained 0.2 or 0.3 cc. of solution of glycoside or of a control solution of appropriately diluted alcohol and the center well contained 0.2 cc. of 20 per cent KOH. The

<sup>2</sup> Preliminary experiments along these lines were carried out by Dr. S. Raska in this laboratory.

vessels were oxygenated for 10 minutes with 100 per cent oxygen during shaking, followed by thermal equilibration for another 10 minutes. Readings were taken every 15 minutes.

The concentrations of components in the medium were: NaCl, 123 mM; KCl, 4.5 mM; CaCl<sub>2</sub>, 0.86 mM; sodium phosphate buffer, 20 mM at pH 7.35; and glucose, 11.1 mM. The glycosides<sup>3</sup> were dissolved in 95 per cent alcohol in a concentration of  $2 \times 10^{-3}$  M and were stored in the cold. The stock solutions were found to keep for several weeks without any signs of deterioration. On the day of the experiment, required dilutions of the glycoside or of alcohol, used in the control runs, were made in distilled water. Since 0.2 or 0.3 cc. of the glycoside or alcohol solution was added to 1.8 or 2.7 cc. of medium, respectively, the final concentrations of the electrolytes or glucose were nine-tenths of those stated above.

Unless otherwise indicated, the glycoside was tipped in 2 hours after the beginning of measured respiration. Respiration was usually measured for a total of 4 hours. In any given run, vessels were set up in duplicate or triplicate, and the experiment as a whole repeated at least three times, usually more. The oxygen consumption has been expressed as mm<sup>3</sup> per 100 mgm. wet weight of tissue.

**RESULTS.** *The effect of scilliroside, digitoxin and ouabain on the respiration of cardiac slices.* When no glycoside was present the oxygen utilization of the cardiac slices decreased about 40 per cent during the first hour to an hour-and-a-half to a level where it was usually maintained without any significant change for the remainder of the second and the third and fourth hours. In order to evaluate the effect of the addition of glycoside at the 2-hour-point, the rates of respiration for the 15-minute periods during the half-hour preceding the introduction of the glycoside were averaged and given an arbitrary value of 100 per cent.

As seen in figure 1, the introduction of scilliroside 2 hours after the beginning of respiration increased the rate of oxygen uptake. The effect and the rapidity with which it occurred were dependent on the concentration of scilliroside. Thus, at a concentration of  $1.6 \times 10^{-8}$  M (1:95,400,000) there was a rise to about 15 per cent above the control value within 75 minutes. Concentrations of  $3.2 \times 10^{-8}$  M (1:47,700,000) and  $1 \times 10^{-7}$  M (1:15,900,000) produced a slow but continuing rise of a slightly greater magnitude which reached its peak in 75 and 60 minutes, respectively. With concentrations of  $1 \times 10^{-6}$  M and  $1 \times 10^{-5}$  M (1:1,590,000 and 1:159,000, respectively), and even greater concentrations, up to  $5 \times 10^{-5}$  M (1:31,000), which do not appear in this graph, the increase in rate of oxygen uptake of about 40 per cent occurred within the first 30 to 45 minutes. The increases in rate of respiration were sustained for the entire period of observation, that is, during the 2 hours following the addition of glycoside. These results differ from those obtained by Lévy et al. (8) and by Wollenberger (13) on rat or guinea pig heart muscle. These investigators, using concentrations similar to those employed here, found that after an initial increase in the rate of respiration, marked decreases set in during the second hour, or earlier, following the addition of glycoside.

The concentration of  $1 \times 10^{-6}$  M scilliroside was apparently the concentration which produced the maximal effects. In another series of experiments, digitoxin, ouabain and scilliroside in this same concentration led to a sustained rise in

<sup>3</sup> Crystalline scilliroside obtained through the courtesy of Sandoz Chemical Works, Inc.

oxygen uptake of the same magnitude and rapidity of effect. The average results are given in table 1.

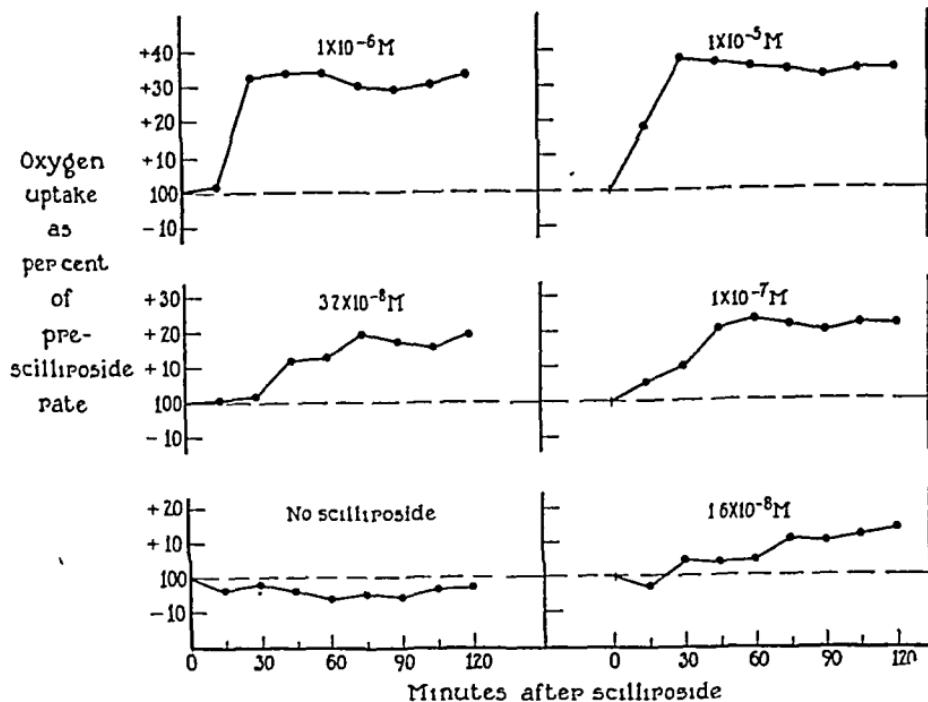


FIG. 1. EFFECT OF CONCENTRATION OF SCILLIROSIDE ON THE RESPIRATION OF SLICES OF CAT HEART. THE VALUES AT EACH CONCENTRATION ARE THE AVERAGES OF 6 EXPERIMENTS

TABLE 1

*Effect of cardiac glycosides on the uptake of oxygen by cat cardiac slices*

Concentration of glycoside was  $1 \times 10^{-6}$  M and of glucose 0.2 per cent. Glycoside or alcohol was introduced 2 hours after the beginning of the experiment. Change in uptake is expressed as per cent of the average uptake in the half-hour immediately preceding the introduction of glycoside, corrected for the change in respiration in control experiments in which alcohol was used. The values recorded below are the averages of 5 experimental runs in duplicate.

GLYCOSIDE	PER CENT CHANGE IN RESPIRATION AT							
	0-15 min	15-30 min	30-45 min	45-60 min	60-75 min	75-90 min	90-105 min	105-120 min
Ouabain	+5	+36	+48	+44	+51	+45	+50	+41
Digitoxin	+6	+39	+50	+47	+41	+49	+48	+44
Scilliroside	+6	+35	+48	+48	+44	+41	+41	+40

*The effect of scilliroside on the respiration of papillary muscle.* By using the papillary muscle of the cat heart, Cattell and Gold (18) were able to demonstrate clearly that ouabain in concentrations of 1 in 5 to 100 million ( $2.61 \times 10^{-7}$  to

$1.31 \times 10^{-8} M$ ) increased the force of contraction of cardiac muscle. It was of interest to us to determine whether or not cardiac glycosides would accelerate the consumption of oxygen by the papillary muscle. Figure 2 shows that a concentration of  $1 \times 10^{-6} M$  (1:1,590,000) of scilliroside caused an acceleration which amounted to about 80 per cent above the control value at the end of 1 to 2 hours after the addition of the glycoside. A concentration of  $3.2 \times 10^{-8} M$  (1:47,700,-000) had no effect on the oxygen uptake.

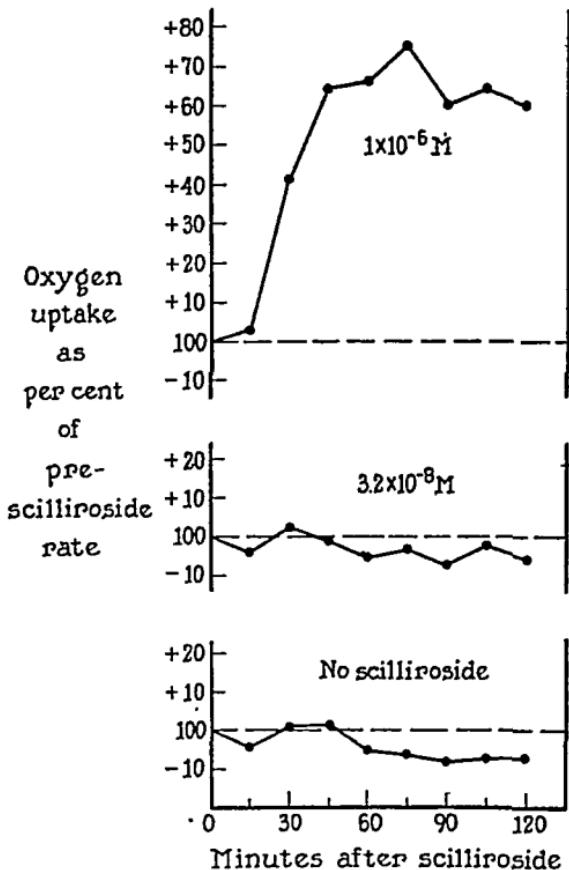


FIG. 2. EFFECT OF CONCENTRATION OF SCILLIROSIDE ON THE RESPIRATION OF PAPILLARY MUSCLE OF CAT HEART

The influence of the concentration of added glucose on the oxygen uptake in the presence of cardiac glycosides. Libert (12) reported that the effect of digitoxin in increasing respiration of cardiac tissue was dependent upon the concentration of added glucose. Thus, at a concentration of 4 mM (0.07 per cent), increases up to about 70 per cent were obtained. At a concentration of 16 mM (0.29 per cent) added glucose, the control respiration was reported to be decreased and the respiration in the presence of digitoxin showed increases, as compared with the

controls, of about 200 per cent. Wollenberger (13) found that the addition of glycoside 1 hour after the beginning of the experiment caused increases in respiration which also were dependent upon the concentration of added glucose. Thus, there was a negligible increase when no glucose was added, 14 per cent increase at 0.02 per cent added glucose and a maximal increase of 35 per cent when the concentration of glucose was 0.2 per cent or higher. Wollenberger, however, reported that these initial increases were followed by a depression amounting to 50 to 60 per cent below the control respiration.

The results here presented (table 2 and figure 3) confirm, in general, the reports that the stimulation in respiration due to the cardiac glycosides is dependent upon the concentration of added glucose. However, the relationship between

TABLE 2

*Effect of cardiac glycosides on uptake of oxygen by cat cardiac slices at various concentrations of added glucose*

The slices were washed in glucose-free medium for 2 periods of 5 minutes each before their addition to the media containing various concentrations of glucose. Concentration of glycoside was  $1 \times 10^{-6} M$ . Glycoside or alcohol was introduced 2 hours after the beginning of the experiment. Change in uptake is expressed as per cent of the average uptake in the half-hour immediately preceding the introduction of glycoside, corrected for the change in respiration in control experiments in which alcohol was used. The values recorded below are the averages of 6 experimental runs in duplicate.

GLUCOSE CONCENTRATION per cent	PER CENT CHANGE IN RESPIRATION AT:							
	0-15 min.	15-30 min.	30-45 min.	45-60 min.	60-75 min.	75-90 min.	90-105 min.	105-120 min.
0.0	+4	+30	+26	+21	+19	+13	-2	-15
0.025	+5	+29	+32	+31	+24	+9	0	-4
0.050	+11	+43	+41	+32	+28	+16	+5	+11
0.100	+16	+46	+46	+42	+43	+35	+28	+24
0.200	+10	+42	+42	+44	+42	+40	+41	+40

such increases and the concentrations of added glucose differ markedly from those reported by the previous investigators.

In this connection the effect of added glucose on the course of respiration of cardiac slices *in the absence of any glycoside* may be described briefly. Libert (12) reported that the  $Q_{O_2}$  was much lower in the presence of 16 mM glucose (0.29 per cent) than in the absence of any added glucose. Wollenberger (13) found that the  $Q_{O_2}$  rose very slightly as the initial concentration of added glucose was increased. In the experiments reported in the present paper the course of oxygen uptake was dependent on the concentration of added glucose. The presence of alcohol in concentrations comparable to those used in the glycoside experiments did not influence the respiration. The addition of glucose at various stages during the course of measured respiration was similarly without effect on the uptake of oxygen.

In the absence of any added glucose, the introduction of scilliroside 2 hours

after the beginning of the experiment led in general to an increase in respiration which reached its maximum, an average of about 30 per cent, within the first half-hour. This was followed by progressively lower rates of oxygen uptake so that by the end of approximately one-and-one-half hours, the respiration was back to the control level and was finally depressed to an average of 15 per cent below the control. At a concentration of 0.025 per cent added glucose the results were similar to those without added glucose in the medium except that the respiration did not decrease so much in the later stages. The maximal increase, about 45 per cent, was obtained at a concentration of 0.05 per cent glucose. This increase was maintained more steadily throughout the course of the experi-

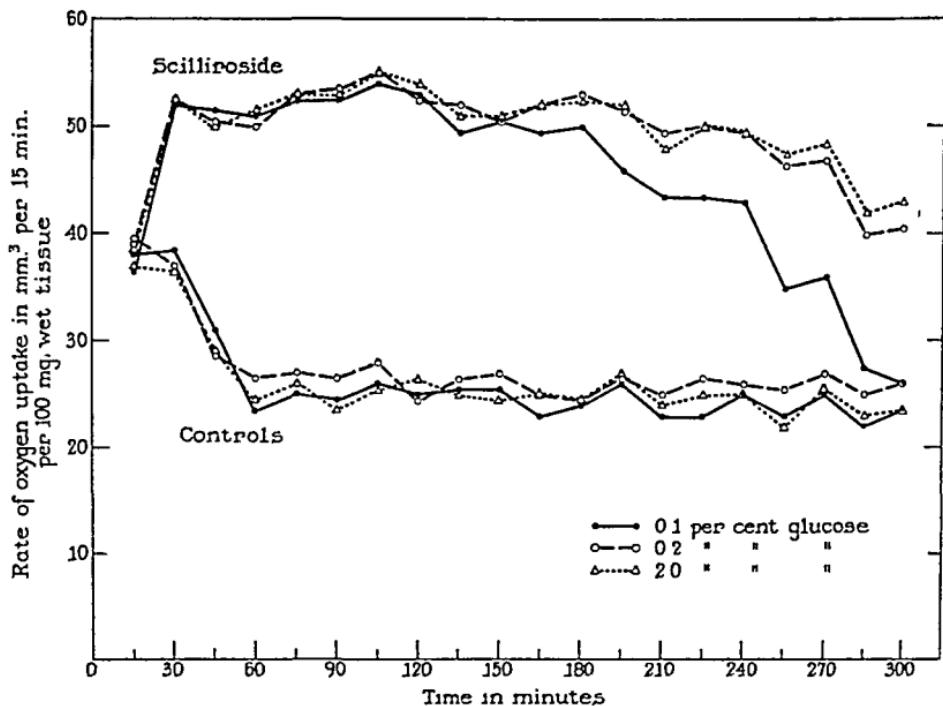


FIG. 3. EFFECT OF THE INITIAL INTRODUCTION OF  $1 \times 10^{-6}$  M SCILLIROSIDE ON THE RESPIRATION OF CARDIAC SLICES AT VARIOUS CONCENTRATIONS OF GLUCOSE

ment, the higher the concentration of added glucose up to a concentration of 0.2 per cent. In experiments containing higher glucose concentrations, 1 and 2 per cent, there was no significant difference from experiments containing 0.2 per cent.

Figure 3 is a typical experiment which shows the effect of scilliroside on respiration at various concentrations of glucose when the scilliroside was introduced not during the course of the experiment but at the beginning. It can be seen that at concentrations of 0.1, 0.2 and 2.0 per cent glucose the respiration increased from an average value of 38 mm<sup>3</sup> to one of 53 mm<sup>3</sup> per 15 minutes per 100 mgm. of wet weight, or about 40 per cent, within the first half-hour. This increased oxygen uptake was maintained for another two-and-a-half hours. Thereafter,

the respiration at the higher concentrations of glucose, 0.2 and 2.0 per cent, decreased slightly, so that at the end of 5 hours the respiration was slightly above the initial rate of oxygen uptake; at a concentration of 0.1 per cent of glucose, the rate of respiration began to decrease so that by the end of 5 hours it was about 30 per cent below the initial rate of respiration. These results differ markedly from those obtained in the control runs which contained, in addition to the usual components, a concentration of alcohol comparable to that present in the glycoside experiments. It may be seen that in these control experiments the initial respiration fell within 1 hour from a value of  $39 \text{ mm}^3$  to one of about  $25 \text{ mm}^3$  per 15 minutes per 100 mgm. wet weight and thereafter continued at this rate, with minor fluctuations, for the remainder of the observation period, or 4 hours after the initial fall. Thus, the rate of respiration in the scilliroside experiments was about 100 per cent higher than in the control experiments. In contrast, as can be seen from table 2, those experiments in which scilliroside was

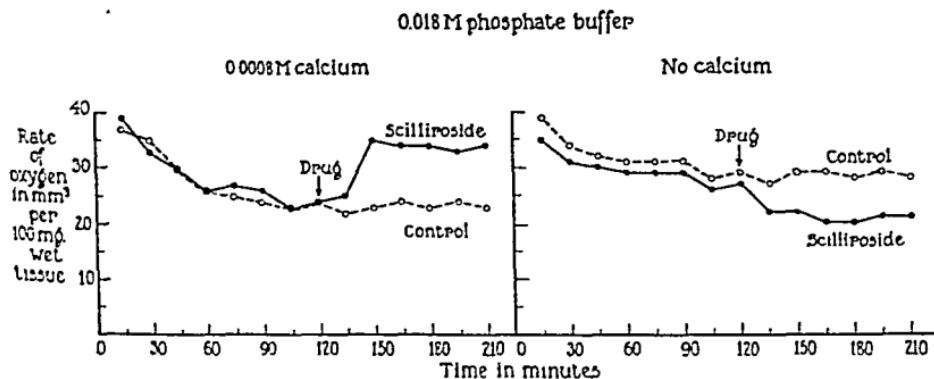


FIG. 4. EFFECT OF CALCIUM ON OXYGEN UPTAKE OF SLICES OF CAT HEART BEFORE AND AFTER THE INTRODUCTION OF SCILLIROSIDE  $1 \times 10^{-6} \text{ M}$

added 2 hours after the beginning of measured respiration, the increase of the rate of respiration was only about 40 per cent above that of the control.

When ouabain or digitoxin was added in the same concentration,  $1 \times 10^{-6} \text{ M}$ , at the beginning of an experiment to a reaction-medium containing 0.2 per cent glucose, the same effect was observed in these as in simultaneously run experiments with scilliroside.

*Influence of calcium ion on the respiration of cardiac slices in the presence of scilliroside.* It should be emphasized that in the experiments so far reported in the present work, calcium ion was present in the media in a final concentration of 0.8 mM. Figure 4 (average results of 6 experiments) shows the effect of adding scilliroside to heart slices when calcium was absent from the medium. It may be noted first that in the initial period and in control runs in the absence of calcium, respiration did not fall off as rapidly nor as much as when calcium was present. The addition of scilliroside, in a concentration of  $1 \times 10^{-6} \text{ M}$ , 2 hours after the beginning of the measured respiration did not produce any increase in the rate of oxygen uptake. Indeed, there was a depression of about 20 per cent

within the first hour. For purposes of comparison, figure 4 shows the accelerant effect of scilliroside when 0.8 mM of calcium was present in the medium. This same depression in the absence of calcium was observed with ouabain or digitoxin in the same concentration as scilliroside.

The influence of the concentration of the calcium ion on the acceleration of respiration by cardiac glycoside was also investigated. It was realized that in order to avoid calcium phosphate precipitation, which occurred when the concentration of calcium was increased to 1.6 mM, it would be necessary to reduce the concentration of the phosphate buffer, even though such a reduction might influence the buffering capacity of the medium. However, as will be seen presently, the reduction of pH during the course of the experiment, as a result of this procedure was found not to influence respiration.

Table 3 shows that at a phosphate concentration of 1.8 mM and a calcium concentration of 0.8 mM the introduction of scilliroside led to a continuous increase

TABLE 3

*Influence of calcium concentration on the accelerant effect of respiration by scilliroside*

Concentration of scilliroside was  $1 \times 10^{-6} M$ , of glucose 0.2 per cent and of phosphate buffer 1.8 mM. Glycoside or alcohol was introduced 2 hours after the beginning of the experiment. Change in uptake is expressed as per cent of the average uptake in the half-hour immediately preceding the introduction of the scilliroside, corrected for the change in respiration in control experiments in which alcohol was used. The values recorded below are the averages of 6 experimental runs in duplicate.

CALCIUM CONCENTRATION mM	PER CENT CHANGE IN RESPIRATION AT:					
	0-15 min.	15-30 min.	30-45 min.	45-60 min.	60-75 min.	75-90 min.
0.8	+8	+53	+77	+88	+97	+71
1.6	+8	+44	+116	+138	+156	+83

in respiration, which by the end of 75 minutes was 97 per cent higher than the control respiration. In contrast, when the concentration of calcium was 1.6 mM the rise was greater, amounting to 150 per cent more than the control respiration at the end of 75 minutes. In these experiments, regardless of calcium concentration, the initial pH was 7.2 and the final pH was 6.7.

*Influence of the concentration of phosphate buffer on the respiration of cardiac slices in the presence of scilliroside.* The relative accelerant effect of scilliroside at a calcium concentration of 0.8 mM and a phosphate concentration of 1.8 mM was 97 per cent above that of its control. It will be recalled that the accelerant effect at the same concentration of calcium but at 18 mM phosphate was only about 50 per cent (table 1). Reference to figure 5 (average results of 6 experiments) shows that in control experiments at lower phosphate concentration there was a decreased oxygen uptake. The respiration at 1.8 mM phosphate concentration preceding introduction of scilliroside was about 50 per cent of that of control experiments in which the concentration of phosphate buffer was 18 mM. Thus, scilliroside exerted a greater accelerant effect on cardiac slices which were

respiring poorly as a result of lowered phosphate in the medium. It was not possible to elevate the rate of respiration by introducing phosphate during the course of respiration so as to raise the concentration from 1.8 mM to 18 mM.

The decreased respiration at the lower phosphate buffer does not appear to be due to a pH effect. The initial pH and the pH at the end of 4 hours of measured respiration in the usual medium containing 18 mM phosphate were, respectively, 7.3 and 6.8. The initial and final pH in the medium containing the lower concentration of phosphate buffer, 1.8 mM, were slightly less, 7.2 and 6.7, respectively. However, the decreased respiration observed in the latter medium could not have been due to these slightly lower pH levels. It was found that in media containing 18 mM phosphate, but initially adjusted to a lower pH, the respiration was the same as in the usual 18 mM phosphate buffer medium. Thus, at a phosphate concentration of 18 mM, with an initial pH of 7.3 and a final pH of 6.8,

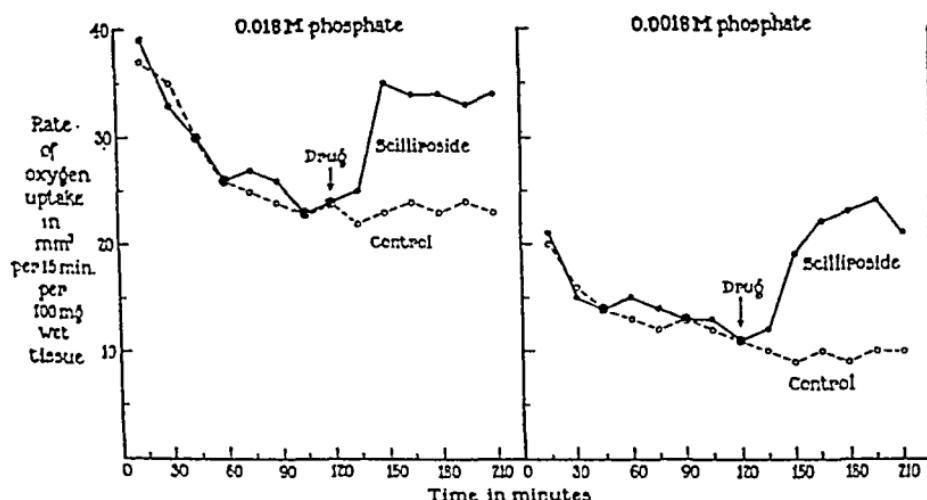


FIG. 5. EFFECT OF PHOSPHATE ON THE OXYGEN UPTAKE OF SLICES OF CAT HEART BEFORE AND AFTER THE INTRODUCTION OF SCILLIROSIDE  $1 \times 10^{-6}$  M

the oxygen uptake was  $45 \text{ mm}^3$  per 100 mgm. of wet tissue during the first 15 minutes of measured respiration and  $28 \text{ mm}^3$  per 100 mgm. of wet tissue during the last 15-minute period ending the fourth hour of measured respiration. Again, at a phosphate concentration of 18 mM, with an initial pH of 7.1 and a final pH of 6.7, the values for respiration for the first and last 15-minute periods were essentially the same as above,  $44$  and  $28 \text{ mm}^3$  of oxygen per 100 mgm. of wet tissue, respectively. When the initial pH was adjusted to 6.9 and the final pH was 6.6, the initial and final uptakes of oxygen were again essentially the same,  $44$  and  $27 \text{ mm}^3$ , respectively, per 15 minutes per 100 mgm. of wet tissue. In contrast, when phosphate concentration was 1.8 mM, with an initial pH of 7.2 and a final pH of 6.7, the initial and final uptakes of oxygen were reduced considerably,  $22$  and  $15 \text{ mm}^3$  of oxygen per 15 minutes per 100 mgm. wet weight of tissue, respectively.

*Influence of magnesium ion on respiration of cardiac slices in the presence of*

*scilliroside.* The use of magnesium ion in respiration media is quite common. Wollenberger (13) and Lévy and her collaborators (8-12) used this ion in their media in studying the effect of cardiac glycosides on respiration. Since we did not routinely use magnesium in our media, a series of experiments was performed in order to determine whether or not this ion had a significant influence on the acceleration of respiration produced by the cardiac glycosides.

It was found that in control runs the presence of 1.08 or 2.16 mM magnesium depressed the respiration of cat cardiac slices slightly, but statistically significantly, during the first 2 hours, but not during the third or fourth hours. Thus, the oxygen uptake during the first hour was 149 mm<sup>3</sup> per 100 mgm. of wet tissue in the absence of magnesium, 128 mm<sup>3</sup> in the presence of 1.08 mM and 120 mm<sup>3</sup> per 100 mgm. wet tissue in the presence of 2.16 mM magnesium<sup>4</sup>. During the second hour the oxygen uptakes were 117, 106 and 98 mm<sup>3</sup> per 100 mgm. of wet tissue, respectively, in the absence of magnesium, in the presence of 1.08 mM and

TABLE 4

*Influence of magnesium concentration on the accelerant effect of respiration by scilliroside*

Concentration of scilliroside was  $1 \times 10^{-6} M$  and of glucose 0.2 per cent. Glycoside or alcohol was introduced 2 hours after the beginning of the experiment. Change in uptake is expressed as per cent of the average uptake in the half-hour immediately preceding the introduction of the scilliroside, corrected for the change in respiration in control experiments in which alcohol was used. The values recorded below are the averages of 4 experimental runs in duplicate.

MAGNESIUM CONCENTRATION mM	PER CENT CHANGE IN RESPIRATION AT:							
	0-15 min.	15-30 min.	30-45 min.	45-60 min.	60-75 min.	75-90 min.	90-105 min.	105-120 min.
0.0	+14	+46	+51	+46	+47	+43	+46	+43
1.08	+11	+37	+58	+66	+71	+62	+54	+58
2.16	+5	+35	+59	+68	+73	+66	+66	+67

in the presence of 2.16 mM magnesium<sup>5</sup>. Although the rate of respiration during the third and fourth hours was lower in the presence of magnesium than in its absence, the differences were not statistically significant.

Table 4 shows that the addition of scilliroside in a concentration of  $1 \times 10^{-6} M$  resulted in the usual increase in respiration, about 40 to 50 per cent in those runs in which magnesium was absent. In the presence of magnesium, the relative accelerant effect was somewhat greater, amounting to a maximal rise of 70 per cent. However, the absolute rate of respiration in the experiments with magnesium was brought up to about the same level as in those without magnesium. It will be recalled that in the absence of calcium the introduction of the glycosides

<sup>4</sup>The "p" values for the differences between the values in the presence of magnesium and in its absence were less than 0.01.

<sup>5</sup>The difference between 106 and 117 gave a "p" value of 0.05; that between 98 and 117 a "p" value of less than 0.01.

caused not an acceleration but a depression in respiration of cat cardiac slices. When calcium was absent, but a concentration of either 1.08 or 2.16 mM of magnesium was present, this characteristic effect due to the absence of calcium was in no wise altered.

**DISCUSSION.** There are not available at the present time adequate data to permit a ready explanation of the accelerant effect of the cardiac glycosides on oxygen uptake by cardiac slices. Wollenberger (13) submitted the hypothesis that the cardiac glycosides increase the permeability of the cell surface to glucose or to other oxidizable substrates. He also explained the inhibition in respiration which he observed to follow the initial acceleration, particularly at high concentrations of glycosides, by this increased permeability which permitted outward diffusion of cellular components essential to respiration. Wollenberger was disinclined to invoke an enzymic mechanism to explain the accelerant effect; he found that ouabain had no effect on the oxygen uptake of homogenized guinea pig heart or of a respiratory enzyme system consisting of glucose dehydrogenase, coenzyme I, cytochrome oxidase, and cytochrome c.

Our results are in accord with Wollenberger's formulation to the extent that we obtain an acceleration of respiration and that the magnitude of the acceleration is dependent, in general, on the concentration of glycoside and added glucose. However, there are several additional assumptions which would have to be made in order to explain our results on the basis that the major action of the glycosides is to increase cell permeability. First, in contrast to Wollenberger, we obtained an acceleration of respiration in the absence of any added glucose. To explain this it would be necessary to assume that in some manner, the endogenous glucose or glycogen is made more readily available for oxidation. Secondly, since we did not observe any inhibition of respiration, it would have to be assumed that, in our experiments at least, the increase in permeability was of sufficient degree to permit readier access of substrate, but not great enough to allow the outward diffusion of essential metabolites. Thirdly, since we found that calcium was essential for acceleration of respiration, the additional assumption would have to be made, that calcium is essential for permeability of the cell surface and that impermeability results when it is absent from the medium. However, such an assumption is contrary to the results of studies which show that decrease in calcium concentration increases cell permeability (19-23). Finally, it is difficult in the light of Wollenberger's theory, to explain the hyporespiration at the lower phosphate concentration, or in the presence of 1.08 or 2.16 mM magnesium ion and the greater relative accelerant effect of the cardiac glycosides under these conditions.

Fenn (24) showed that contractures produced in frog's sartorius muscle by various chemical agents, notably caffeine, were accompanied by increases in oxygen consumption. The question arises whether or not the increased oxygen consumption of cat heart slices in the presence of cardiac glycosides is associated with contracture of the muscle. No direct evidence concerning this point exists. Cattell (25) found that cat papillary muscle showed no contractures in the presence of ouabain ranging in concentration from 1 in 5 to 70 million ( $2.61 \times 10^{-7}$  to  $1.87 \times 10^{-8}$  M).

## SUMMARY

1. The oxygen consumption of cat cardiac slices of the left ventricle was measured and the changes produced under the influence of various ions and cardiac glycosides were observed.

2: The introduction of scilliroside in concentrations of  $1.6 \times 10^{-8}$  to  $5 \times 10^{-5} M$  two hours after the beginning of measured respiration produced a maintained increase in the rate of oxygen uptake. The effect and rapidity with which this increase occurred were dependent on the concentration of scilliroside. A concentration of  $1 \times 10^{-5} M$  produced maximal effects. Digitoxin and ouabain in this concentration produced similar results. The oxygen consumption of the papillary muscle of the right ventricle was accelerated by a concentration of  $1 \times 10^{-6} M$ , but not by a concentration of  $3.2 \times 10^{-8} M$  scilliroside.

3. The degree of acceleration of respiration increased as the concentration of added glucose was increased up to 0.2 per cent.

4. The presence of calcium ion was essential to the acceleration of respiration by the cardiac glycosides. When calcium was absent from the medium, the introduction of cardiac glycosides caused a decrease in respiration of cardiac tissue.

5. At a low phosphate concentration the initial respiration was approximately 50 per cent lower than usual. The introduction of scilliroside led to a 75 per cent increase in the rate of oxygen uptake, compared to a 50 per cent increase at higher phosphate concentration. When the concentration of phosphate was low and that of calcium was high the introduction of scilliroside led to a relative acceleration of 150 per cent in the rate of oxygen uptake.

6. Tissues in a medium containing magnesium in concentrations of 1.08 or 2.16 mM, consumed initially less oxygen than when magnesium was absent. The introduction of scilliroside led to a greater relative acceleration in uptake in the presence of magnesium.

7. Hyporespiring tissues showed a relatively greater acceleration in respiration after the introduction of scilliroside than did normally respiring slices.

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# STUDIES ON THE LAXATIVE ACTIVITY OF TRIPHENYL-METHANE DERIVATIVES

## I. RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY OF PHENOLPHTHALEIN CONGENERS

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With the synthesis of phenolphthalein in 1871 (1) Von Baeyer opened a new field of triphenylmethane derivatives, the diphenylphthalides. Therapeutists, by an accident (2), established the capacity of phenolphthalein to produce laxation, a property which has been used and abused in hundreds of proprietary anticonstipants consumed in billions of doses annually. However, all such proprietaries contain only one representative of the class, phenolphthalein (PTH) itself, and virtually no other diphenylphthalide has ever found serious therapeutic application. This is due to the lack of pharmacological studies of the cathartic potentialities of PTH congeners.

The relationship between the chemical structure of the congeneric triphenylmethanes and their laxative activity has received attention only recently. A comparative study of the laxative potency of a series of eight congeners and a few derivatives has been reported (3). The number of PTH homologs tested has now grown to fifteen, that of analogs to nine and that of derivatives to twenty, and data obtained previously have been either verified or amended by additional assays. The present communication is concerned with these studies and with the problem of structure-activity relationship in this class.

**EXPERIMENTAL PROCEDURE.** The laxative activity of PTH and 46 congeners was determined by bioassay in the rhesus monkey as described in previous papers (3, 4, 5, 6; see also 7<sup>1</sup>). The reference standard was a sample of U. S. P. phenolphthalein, the potency value (P) of which was established in over 4000 experiments.<sup>2</sup>

The substances studied are chemically characterized in table 1 and listed in columns 1 to 5 of tables 2 and 3. They were prepared by Dr. M. Hubacher.<sup>3</sup> For the majority of substances, the synthesis followed procedures described in the chemical literature. On occasion new syntheses were employed and new or decisively improved products obtained. The test substances (see tables 2 and 3; general formula (I) in table 1) differ from PTH either in the composition of one or both of the two phenol rings R<sup>1</sup> and R<sup>2</sup> or in that of the R<sup>2</sup>:R<sup>4</sup> ring system, and can be grouped as follows:

*A. Homologous diphenylphthalides.* Group 1.—The series of isomeric and homologous mono- or polyhydric diphenylphthalides proper, in which R<sup>1</sup> and R<sup>2</sup> are benzene rings with varying numbers of OH-groups in different positions. Group 2.—Derivatives of Group 1, in which phenolic hydroxyl groups are blocked by formation of ethers or esters, including formation of fluorans. Group 3.—Polyhydric diphenylphthalides with additional substituents other than hydroxyl groups at the rings R<sup>1</sup> and R<sup>2</sup>.

<sup>1</sup> Monkeys were supplied by Exlax Inc., Brooklyn, New York.

<sup>2</sup> For greater convenience, the potency of phenolphthalein is defined as 100 instead of the previously used value of 1.0.

<sup>3</sup> Chief Chemist, Exlax Inc., Brooklyn, New York.

TABLE I

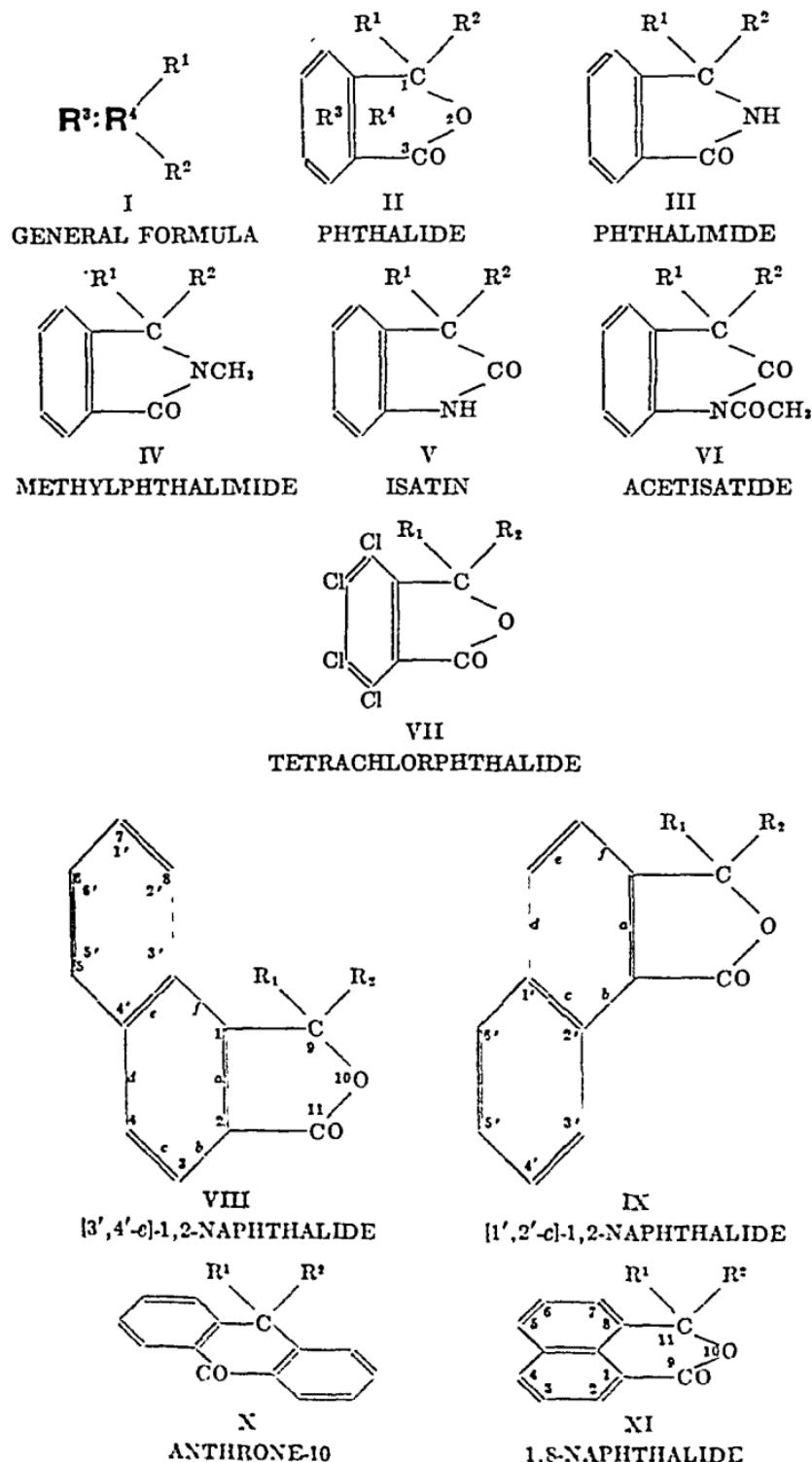


TABLE 2  
Laxative activity of diphenylphthalides

NO.	SUBSTANCE	POSITION OF OH-groups AT RING		M.P. (CORN.)	NUMBER OF ASSAY EXPERIMENTS [Per unit of wt.]	MEAN LAXATIVE POTENCY [Per mole]
		R <sup>1</sup>	R <sup>3</sup>			
Group 1						
1	Phthalophenone	—	—	115.0-115.3	9	7.25
2	Phenolbenzenephthalein	4	—	171	25	0.85
3	Phenolphthalein	4	4	261-262	4000	100.00
4	Pyrocatecholbenzenephthalein	3, 4	—	168.1-169.3	6	30.00
5	Resorcinolbenzenephthalein	2, 4	—	287.7-289.4	65	<1.50
6	Hydroquinonebenzenephthalein	2, 5	—	243.3-246.5	15	<1.64
7	Isophenolphthalein	2	4	205.4-206.1	19	<11.60
8	Pyrogallolbenzenephthalein	3, 4, 5	—	191.8-193.7	31	173.00
9	Phenopyrocatecholphthalein	3, 4	4	205-209	21	68.50
10	Hydroxyhydroquinonebenzenephthalein	2, 3, 5	—	—	7	<3.50
11	Phloroglucinolbenzenephthalein	2, 4, 6	—	90-110	10	<1.23
12	Phenolisopyrocatecholphthalein	2, 3	4	270-275	5	<6.20
13	Phenoxyresorcinolphthalein	2, 4	4	190-200	9	1.00
14	Phenolpyrogallolphthalein	3, 4, 5	4	235-237	7	47.00
15	Pyrocatecholphthalein	3, 4	3, 4	95-125	11	3.30
16	Phenolphloroglucinolphthalein	2, 4, 6	4	—	5	<1.00



TABLE 3  
*Laxative activity of analogs of the phthalides*

CURRENT NO.	NO. IN TABLE I	SUBSTANCE	R <sup>1</sup> :R <sup>4</sup>	POSITION OF OH-GROUPS ATRING	M. P. (CORR.)	NUMBER OF ASSAY EXPERIMENTS	MEAN LAXATIVE POTENCY	
							Per unit of weight	Per mole
1	II	Phenolphthalein		4-OH	261	4000	100.00	100.00
2	VII	Phenoltetrachlorphthalein		4-OH	318	320	<5.00	<7.16
3	VIII	1,2-Phenolnaphthalide		4-OH	291	292	10	47.00
4	IX	1,2-Phenoquinophthalide, iso- Phthalimide		4-OH	267	269	6	56.60
5	III	Phenolphthalimide		4-OH	267	-270	8	9.94
6	IV	N-methylphenophthalimide		4-OH	259	-263	5	9.56
7	V	Diacetylphenolsatin	4-O-Ac	240	96	1730.00	2176.00	
8	VI	Triacetylphenolsatin	4-O-Ac	—	4	1040.00	1353.00	
9	X	9,9-Di(4-hydroxyphenyl)- anthrone-10	4-OH	310	3	47.00	57.70	
10	XI	1,8-Phenolnaphthalide	4-OH	271	-272	36	500.00	592.00
11	—	Phenolbenzene-1,8-naphthalide	4-OH	229.9-232.0	5	2.50	2.76	
12	—	Pyrogallolbenzene-1,8- naphthalide	3,4,5-OH	307	-312	10	<11.00	<13.50
13	—	Pyrogallolphenol-1,8- naphthalide	3,4,5-OH	173	-197	4	<5.60	<8.68

*B. Analogs of the diphenylphthalides.* The analogs studied belong to three groups (see tables 1 and 3) according to whether they differ from the phthalides (II) in R<sup>3</sup> (Group 4; VII, VIII, IX), in R<sup>4</sup> (Group 5; III, IV, V, VI, X), or in both rings (Group 6; X of the fused 2-ring system).

The results of bioassay are reported in tables 2 and 3. The mean value of laxative potency (P) is given on the basis of weight (column 7) and molecular weight (column 8). Whereas under favorable assay conditions the mean value obtained with the method of intra-individual determination (3, 4, 5, 6; see also 7) is reliable within a range of  $\pm 10$  per cent or less, the necessary number of experiments in the critical range was limited in some of the assays either by feeble activity of the test substance (due to which a considerable part of the limited supply of drug was employed in a few large-dose experiments), or by the taste of the substance (due to which only a few monkeys could be induced to consume the requisite dose). In the majority of experiments no attempt was made to find the range of effectiveness when the potency was less than  $\frac{1}{5}$  that of PTH. For these reasons, the reliability of each potency value is indicated as previously (3) by recording, in column 6, the number of assay experiments performed.

**RESULTS.** *A. Homologous diphenylphthalides.* Group 1. As shown in table 2 and illustrated by figure 1, in the group of polyhydric diphenylphthalides proper (Nos. 1 to 16), one of the isomeric trihydroxyl derivatives has the highest potency; it is the trihydric pyrogallolbenzenephthalein (No. 8) which is superior to phenolphthalein by 63 per cent. The series contains two additional members having about one-half or more the potency of PTH, one trihydric (No. 9; potency P = 68.5), the other tetrahydric (No. 14; P = 47). Next in order of decreasing potency is a dihydric derivative (No. 4; P = 30).

The remaining eleven representatives had less than 11.6 per cent of the activity of PTH. Prominent among these is the hydroxyl-free parent substance (No. 1; P = 7.25). At least eight substances representing mono- to tetrahydric derivatives had less than 5 per cent, and at least six substances even less than 2 per cent of the potency of PTH.

Group 2. Ethers as well as esters of the polyhydric compounds of Group 1 were generally less potent than the corresponding free phenols. Among the esters, the diacetate of PTH (No. 23) was more potent than the dibenzoate (No. 24). Gradual reduction of the number of free alcohol groups in the trihydric pyrogallolbenzenephthalein (No. 8) resulted in a gradual decrease in laxative potency, as shown in tables 2 and 4.

By etherification, laxative potency was decreased to a varying degree. The glucosidic phenolphthalein ether (No. 25) still possessed almost the potency of its PTH moiety. Dimethylation of PTH diminished the potency by 88 per cent (table 1, No. 22). On the other hand, monomethylation of PTH (No. 21) reduced the potency more than did dimethylation, whereas methylation of the monohydroxyl homolog resulted in an ether (No. 20) that had three times the potency of the free alcohol.

All fluorans tested were found devoid of demonstrable laxative activity. If they have any laxative action, their potency is less than  $\frac{1}{5}$  that of PTH.

Group 3. Any alkyl (Nos. 30 to 33) or aryl substitution (Nos. 34 and 35) on C-atoms of R<sup>1</sup>, R<sup>2</sup> or both resulted in a great reduction in laxative activity.

FIGURE 1

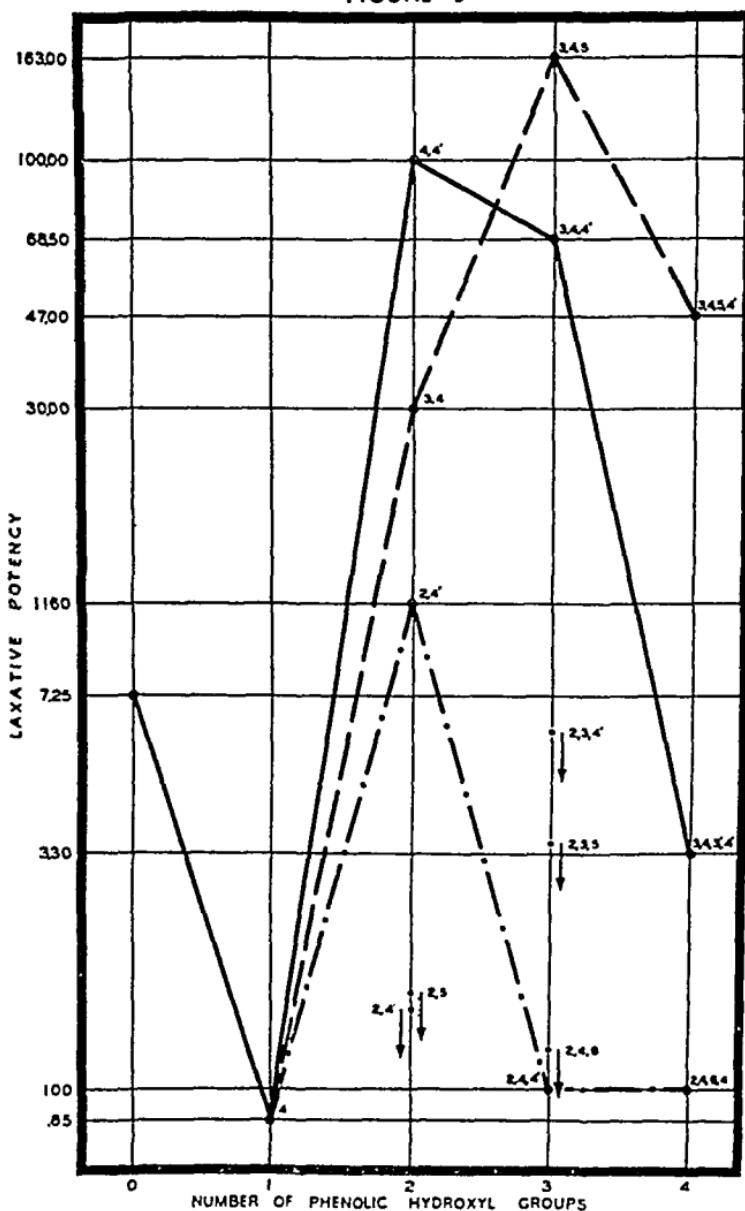


FIG. 1. VARIATION OF LAXATIVE POTENCY OF POLYHYDRIC DIPHENYLPHTHALIDES (ORDINATE; NUMBERS AT MARGIN = POTENCY; YARDSTICK: LOG POTENCY) WITH NUMBER OF PHENOLIC HYDROXYL SUBSTITUENTS (ABSCISSA)

—: series formed by progressively adding an OH group in the most distal position vacant. - - - : series formed by progressively adding a p- or m-OH group. - - - - : series of mixed p- and o-OH homologs. Figures at projection points signify position of the OH groups of the compound tested.

Introduction in PTH of a single methyl group in position 3 (No. 30) reduced the potency from 100 to 12.5. Introduction of two methyl groups in positions 3 and 3' (No. 31), of more and larger alkyl groups (Nos. 32 and 33), of a phenol group (No. 34), or of a conjugated benzene ring (No. 35) abolished any laxative activity demonstrable by the assay method.

*B. Analogs of phenolphthalein.* Group 4. In the three analogs of this group, the benzene ring R<sup>3</sup> of phthalide is replaced by tetrachlorobenzene (VII; table 3, No. 2) or by naphthalene (VIII, IX; Nos. 3 and 4). The first analog, phenol-tetrachlorophthalein, was inactive. The second two, the isomeric 1,2-naphthalenedicarboxylic anhydrides, were also considerably less potent than PTH.

Group 5. In the five analogs of this group, the hydrofuran ring R<sup>4</sup> is replaced by pyrrolidine (III, No. 5; V, No. 7), N-methyl-pyrrolidine (IV, No. 6), acetopyrrolidine (VI, No. 8) or naphthalene (X, No. 9). The two pyrrolidine analogs,

TABLE 4

*Change in laxative potency by gradual acetylation of phenolic hydroxyl groups*

Free alcohol	3,4,5-TRIHYDROXY-DIPHENYLPHTHALIDE		
	Mono-	Di-	Tri-
	Acetate		
Substance No.....	8	26	27
Number of free OH-groups...	3	2	1
Potency.....	163.0	23.0	15.6
			2.5

although their R<sup>3</sup>:R<sup>4</sup> skeletons are isomeric, represent diametrical extremes in laxative potency. The phthalimide derivative No. 5 had only about one-tenth, but the isatin derivative No. 7 had more than 17 times the potency of PTH. In both types of pyrrolidine analogs of PTH, N-substitution decreased the activity; in the case of the isatin derivative No. 8, however, the potency was still ten times that of PTH. The laxative activity of the R<sup>4</sup> naphthalene analog of PTH, No. 9, was almost one-half that of PTH.

Group 6. In the examples of this group, the benzene ring R<sup>3</sup> is replaced by naphthalene and the hydrofuran ring R<sup>4</sup> by a hydropyran ring. Phenolnaphthalene (XI; No. 10) had five times the potency of PTH. The three other examples studied in this group were polyhydric homologs of phenolnaphthalene, differing from the latter and from each other in the number and position of phenolic hydroxyl groups. All three (Nos. 11, 12 and 13) were greatly inferior in potency to both 1,8-phenolnaphthalene and PTH.

**DISCUSSION.** In a previous study of the SAR<sup>4</sup> of aromatic laxatives (3), some data were presented indicating that polyhydric di-, tri- and tetraphenylmethanes become laxophoric<sup>5</sup> when connected with an additional carbonyl containing ring. Moreover it was suggested that in the class of diphenylphthalides the laxative

<sup>4</sup>The abbreviation "SAR" (structure-activity relationship) is used to express the lengthy phrase "relationship between chemical configuration and biological activity."

potency varies with the number of phenolic hydroxyls, and that a closer study of the influence of this variable might answer the question whether PTH is the most effective representative of the series of these congeners. The present study helps to elucidate these aspects of the SAR.

*A. SAR of the homologous polyhydric diphenylphthalides.* According to the bioassay of laxative potency of 35 congeners and derivatives, SAR in the class of diphenylphthalides proper appears to conform to the following rules:

1. The parent substance of the class, the OH-free diphenylphthalide (No. 1), possesses considerable laxative activity.

2. Introduction of a single hydroxyl group into the parent substance decreases the potency.

3. Two or more phenolic OH groups are auxocathartic,<sup>5</sup> but only when they are in a position distal to the phthalide ring (positions 3, 4, 5). Under these conditions, laxative potency increases with the number of phenolic hydroxyl groups up to a maximum embodied in one of the isomeric trihydric derivatives, and decreases with further increase in the number of OH-groups.

4. In contrast, proximal OH-groups, in positions 2 and 6, are miocathartic.<sup>5</sup>

5. The laxative potency of homologs with more than one hydroxyl is also decreased (a) by blocking of free phenolic hydroxyl groups, both ether- and esterification being miocathartic, (b) by internal etherification between proximal OH-groups of the two phenol rings which gives rise to formation of a fourth (pyran) ring characteristic of the fluorans, and (c) by any alkyl or aryl substituents in R<sup>1</sup> or R<sup>2</sup>.

The details of SAR disclosed by the bioassay findings and outlined by the above rules cannot be discussed here exhaustively and will be dealt with more extensively elsewhere. Only some major structural requirements for laxative activity in this homologous series may be reviewed.

Uninhibited rotation of the rings R<sup>1</sup> and R<sup>2</sup> is a primary prerequisite, as indicated by the inactivity of the fluorans and the miocathartic role of proximal substituents, including proximal OH groups. The positional prerequisite for auxocathartic influence of OH groups, namely, their distal position, puts emphasis on the significance of location of these groups near the periphery of the molecule. The prerequisite of number of OH groups, namely, a minimum of two and an optimum of three, suggests that hydroaffinity favors laxative activity, if at all, only within certain limits and that particularly in tetrahydric and higher homologs there may be interference between hydrophilic and auxocathartic influences of the OH groups. The miocathartic role of a solitary distal OH group is difficult to understand; the fact, however, explains why an otherwise miocathartic blocking of this group increases (Nos. 20 and 22) and, contrariwise, unblocking decreases the potency (compare No. 22 with No. 21).

Symmetrical arrangement of the substituents at the phenol ring or equality of substitutions of the two rings are not prerequisites of higher potency. To the

\* The term "laxophoric", often used somewhat indiscriminately, will be limited to designate properties of the structural skeleton, which are correlated with cathartic activity. Substituents which increase laxative activity will be called "auxocathartic", whereas substituents which decrease laxative activity will be called "miocathartic."

contrary, the majority of potent homologs and especially the most potent representative are intra- and internuclearly asymmetrical. This implies that they were tested as racemates which may be less potent by 50 per cent than one of their optical isomers; the maximum potency in the class may thus be as high as 326.

The trend of potencies of the substances studied out of the 190 compounds of the entire class is not suggestive of a likelihood of higher potencies in nontested substances, as will be shown elsewhere.

*B. Analogs of phenolphthalein.* All analogs studied comply with the previously (3) derived requirements for laxophoric character of the skeletal  $R^3:R^4$  ring system. The third benzene ring of the triphenylmethane is always fused into a two-ring system containing the triphenyl-substituted methenyl group and an oxo group. The following SAR trends are indicated.

Substitutions in the benzene ring  $R^3$  of the phthalide ring system decreased (Nos. 3 and 4) or abolished (No. 2) laxative activity. The two isomeric naphthalene derivatives differ in potency and in the distance of the phenyl groups from the perimeter of the molecule. Structural alterations in ring  $R^4$  of the two-ring system, quite contrary to those in ring  $R^3$ , can result in a remarkable increase in laxative potency. The isomeric substances studied differ chemically as much as they do in pharmacological activity, the weakly potent phthalimides being dicarboximides, the highly potent isatins, monocarboxanilides. The conversion of phthalide into phthalimide is not irreversible, particularly not at gastric pH. The insignificant laxative activity of the dihydroxydiphenylphthalimide, No. 6, may therefore be due to reconversion of about 10 per cent of the imide to the phthalic anhydride. The dihydroxydiphenolisatins, on the other hand, are distinguished by extremely low solubility and great chemical stability. The outstanding cathartic activity of the isatin derivative No. 7 is the more remarkable inasmuch as the two phenolic hydroxyl groups are blocked by acetylation, an alteration which, in the phthalides, results in a very considerable decrease in potency. It appears indeed as if the free phenol, dihydroxydiphenylisatin, is still more potent than its diacetate (8). Alteration of the structure of both rings of the  $R^3:R^4$  ring system can also give rise to a marked increase in laxative potency. The pyran derivative phenolnaphthalein (No. 10) differs from its furan isomers (No. 3, P = 47.0; and No. 4, P = 8.25), by having a potency of 500. The potencies of the four homologous derivatives (table 3, Nos. 10, 11, 12 and 13) of the parent substance, diphenylnaphthalide, when compared with those of the corresponding polyhydric derivatives of diphenylphthalide (table 2, Nos. 2, 3, and 8), indicate that the rule for maximum auxocathartic influence of distal OH groups in diphenylphthalides does not apply without modification to 1,8-naphthalides.

The question whether the leading role in medicinal use played by PTH is justified from the aspect of potency is clearly answered by this study. At least one, if not more, of the homologs and three analogs of PTH possess much greater potency than PTH. The highest laxative potencies were embodied in analogs with varied  $R^3:R^4$  ring system. Whereas more types of analogs will have to be studied before the correlation of potency with the skeletal structure can be elu-

citated to a similar extent as that with the phenolic hydroxyl substituents in the diphenylphthalide series, the data obtained can lend guidance and obvious clues in the search for potent cathartics.

As to the mechanism of laxative action, the variable role of the phenolic hydroxyl groups, as well as the laxophoric character of OH-free congeners and the strong miocathartic influence of the fluoran ring closure, refute the older hypotheses. Laxative activity in this class is not a "local irritant phenol action", nor can it be related to quinoid structure. The quinoid form requires a pH which does not exist in the body, and fluorans were devoid of laxative activity despite their aptitude for quinoid conversion and the presence of distal OH-groups (see Nos. 18 and 19).

#### SUMMARY

1. By a study of the laxative potency in the monkey of 46 isomers, homologs and analogs of phenolphthalein, some light is shed upon the structural factors which vary the laxophoric character of the basic two-ring skeleton and govern the modification of cathartic activity by substituents.

2. In the class of isomers and homologs of phenolphthalein, the variation of laxative activity by hydroxyl and other substituents at the free benzene rings, and of blocking of hydroxyl groups, could be expressed by a series of rules which are compatible with certain concepts of spatial arrangement and intramolecular kinetics. Maximum potency in the series, 1.63 times that of phenolphthalein, was embodied in the racemic trihydric pyrogallolbenzenephthalein, which suggests the possibility of still higher potency in an optical isomer.

3. In the analogs of phenolphthalein, the laxophoric character of the skeletal two-ring system was decreased in those compounds studied in which the benzene part of the skeleton was enlarged by substitution, and when the phthalide was replaced by phthalimide, N-methylphthalimide or anthrone, but greatly increased when it was replaced by isatin, N-acetylisatin or naphthalide. Phenol-naphthalein had 5 and the isatins up to 17 times the potency of phenolphthalein.

4. Reasons are presented for discarding certain older views concerning the mechanism of laxative action of phenolphthalein and its congeners.

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# A COMPARISON OF NINE LOCAL ANESTHETICS

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In general, the inherent safety of a drug increases with the ratio of the toxic dose or concentration to the effective dose or concentration. A definite advance in defining such ratios was made by McIntyre and Sievers (1) when they introduced the concept of an Anesthetic Index. The name hardly does justice to the main principle of this index, namely, that it is a relative rating. Absolute figures for toxicity and potency are not used to calculate this ratio, but toxicity relative to procaine and potency relative to procaine determined by experiments done in the same manner on the same species with the drug and with procaine. In some applications, such as surface anesthesia, it would be more relevant to use cocaine as a drug of reference rather than procaine and for different applications different methods should be used for the estimation of relative potency and relative toxicity. Therefore, a new series of indices is proposed, called Relative Ratings (R.R.). For each the drug of reference should be named, e.g. the Relative Rating with reference to cocaine should be designated R.R. (cocaine). In addition the bases of comparison should be specified in tables or in the text. Example for any drug X:

$$\frac{\text{L.D. 50 of cocaine}}{\text{L.D. 50 of } X} = \text{Relative Toxicity (cocaine) or Toxicity relative to cocaine.}$$

$$\frac{\text{E.C. 50 of cocaine}}{\text{E.C. 50 of } X} = \text{Relative Potency (cocaine) or Potency relative to cocaine.}$$

Then

$$\frac{\text{Relative Potency (cocaine)}}{\text{Relative Toxicity (cocaine)}} = \text{Relative Rating (cocaine).}$$

This particular R.R. would seem to be appropriate for the comparison of drugs for topical application to mucous membranes.

Obviously for different uses such as topical application, nerve block, and infiltration, the methods of measuring relative potency and relative toxicity should be different. For example, in corneal anesthesia or a nerve block of a small region as used in dentistry, systemic toxicity is rarely a problem and it would be more useful to know the relative toxicity for the tissues at the site of injection or application. Accordingly some experiments were performed in an attempt to establish several Relative Ratings for nine local anesthetics.

Most of the substances used are well known as local anesthetics and descriptions of them appear in the British Pharmacopoeia, the United States Pharmacopoeia or New and Non-Official Remedies. Pontocaine is known in the B.P. as Amethocaine and in the U.S.P. as Tetracaine. Nupercaine is known in the

citated to a similar extent as that with the phenolic hydroxyl substituents in the diphenylphthalide series, the data obtained can lend guidance and obvious clues in the search for potent cathartics.

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in millimeters, an average of several diameters being taken. If the area was irregular in outline.

A number of tests were done on the effect of epinephrine on the toxicity of the anesthetics for the skin. Three concentrations of each anesthetic (0.5, 1.0 and 2.0 per cent) were prepared with epinephrine (1:10,000) and injected intradermally on one side of the midline of the back area. On the other side, the same concentrations of the same drugs without epinephrine were administered. As some of the drugs were quite toxic the volume per wheal was limited to 0.1 cc. Even so, those animals receiving pontocaine and nupercaine developed convulsions which were controlled by the intraperitoneal injection of 0.2 cc. of Dts! (Ciba) liquid compound.

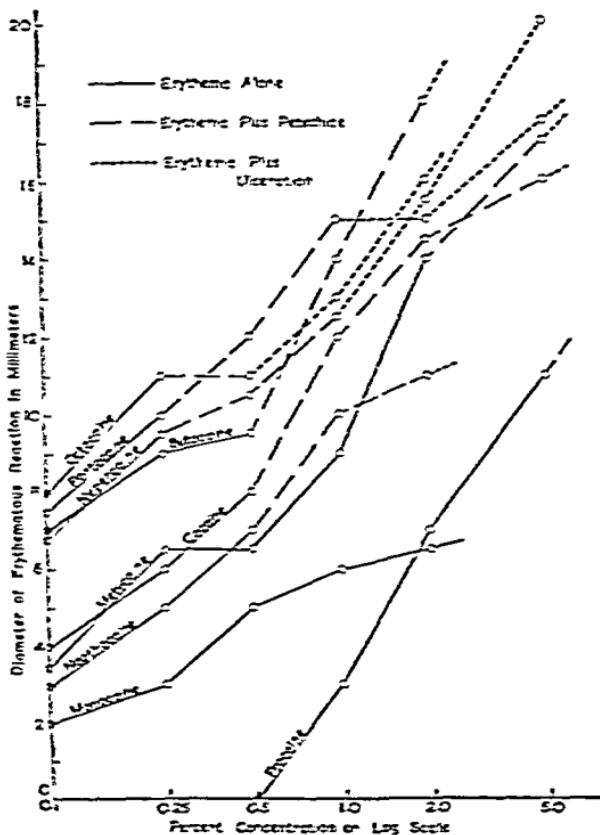


FIG. 1. TISSUE TOXICITY OF LOCAL ANESTHETICS BY INJECTION OF 0.2 CC. INTRADERMALLY IN GUINEA PIGS. REACTIONS READ 24 HOURS AFTER INJECTION

**RESULTS.** Epinephrine had little effect on the tissue toxicity of the local anesthetics tested, except in cases of octacaine, pontocaine, and nupercaine, where the toxic reaction seemed to be somewhat increased.

In the experiments with the local anesthetic alone, in which more concentrations were tested, the average diameter of the erythematous area increased in approximately linear proportion to log concentration. A straight line was drawn by inspection to represent the relation for each drug and this line was used in the calculation of relative toxicity. In the case of monocaine it was necessary to extrapolate from the data. The actual observations are shown in fig. 1, which

B.P. as Cinchocaine and in N.N.R. as Dibucaine. Naphthocaine is a relatively new drug with the chemical name of beta-diethylaminoethyl-4-amino-1-naphthoic acid (2). Octacaine is also relatively new and is 2-(1-methyl heptyl)2,2-dimethyl ethyl para-amino benzoate (3). All the drugs except butacaine sulfate were used in the form of the hydrochloride salts.

**SYSTEMIC TOXICITY. Method.** The toxicity was determined by injecting the different drugs intraperitoneally into male white mice weighing 18-24 grams. For each anesthetic at each dose level not less than 10 mice were injected with amounts calculated on the basis of body weight. The animals were kept under observation at an environmental temperature of 70-78°F. for 24 hours. Sufficient variation in dosage for each drug was used so that less than half the animals died in some groups of mice and more than half died in other groups.

The mortality rates in probit units were plotted against log dose in mgm./kgm. A straight line was drawn by inspection. The dosage at the point on this line corresponding to probit 5 was taken as log L.D. 50. The standard errors of the L.D. 50's were estimated by the method of first approximation, as described by Finney (4).

TABLE I  
L.D. 50's for nine local anesthetics

DRUG	L D 50 ± S E	L D 50 ± S E WITH EPINEPHRINE	DIFFERENCE	P OF DIFFERENCE
	mgm /kgm	mg /kgm		
Procaine	185 ± 4.0	153 ± 4.6	-17	<0.01
Metycaine	182 ± 11.0	137 ± 4.0	-25	<0.01
Monocaine	175 ± 5.9	160 ± 4.2	-8	>0.05
Naphthocaine	118 ± 3.8	125 ± 3.6	+6	>0.05
Butacaine	85 ± 5.5	76 ± 2.2	-10	>0.05
Octacaine	81 ± 3.2	77 ± 1.1	-5	>0.05
Cocaine	67 ± 4.7	73 ± 2.6	+9	>0.05
Pontocaine	52 ± 2.1	85 ± 0.7	+63	<0.01
Nupercaine	29 ± 1.7	38 ± 0.8	+31	<0.01

The effect of epinephrine on the lethal dose was also tested for each drug. Epinephrine was added in the proportion of 1 mgm. per gram of the local anesthetic, except for the very potent drugs nupercaine, and pontocaine, for which the ratio of 2 mgm. of epinephrine per gram of drug was used.

**RESULTS.** The results are presented in table I. It will be noted that with procaine and metycaine, epinephrine increased the toxicity by a small but significant fraction, while with pontocaine and nupercaine, epinephrine decreased the toxicity.

**TISSUE TOXICITY. Method.** As studies on the toxicity of local anesthetics for tissues have not been numerous, the following experiments should be regarded as exploratory and tentative. Using one animal for each drug, intradermal injections of 0.2 cc. in concentrations of 0.1, 0.25, 0.5, 1.0, 2.0 and, if solubility permitted, 5.0 per cent, all in 0.9 per cent NaCl, were made on the back of the guinea pig. Control injections of normal saline were made for comparison. Reactions, consisting of varying degrees of erythema, petechiae and ulceration with crusting were recorded one-half hour, one hour, four hours, three days, one week and three weeks following injection. At the height of the reaction, which occurred about 24 hours after the injection in all cases, the diameter of each erythematous area was measured

important difference which is shown in fig. 2. The slope for metycaine is definitely less than that for the others and this characteristic is also apparent in fig. 3. From the lines in fig. 2 an E.C. 50 for any duration of complete anesthesia can be read off, because each point on the line represents an average duration at that concentration. By an approximate estimation the S.E. of such an E.C. 50 is not more than 10 per cent of the value. This is applicable to all the drugs with the exception of metycaine, for which the S.E. is about 16 per cent.

The relative potencies of the various drugs are shown in table II (procaine = 1).

**TOPICAL ANESTHESIA.** Due to its accessibility and the presence of reliable reflex action, the cornea has been used almost exclusively for assessing surface

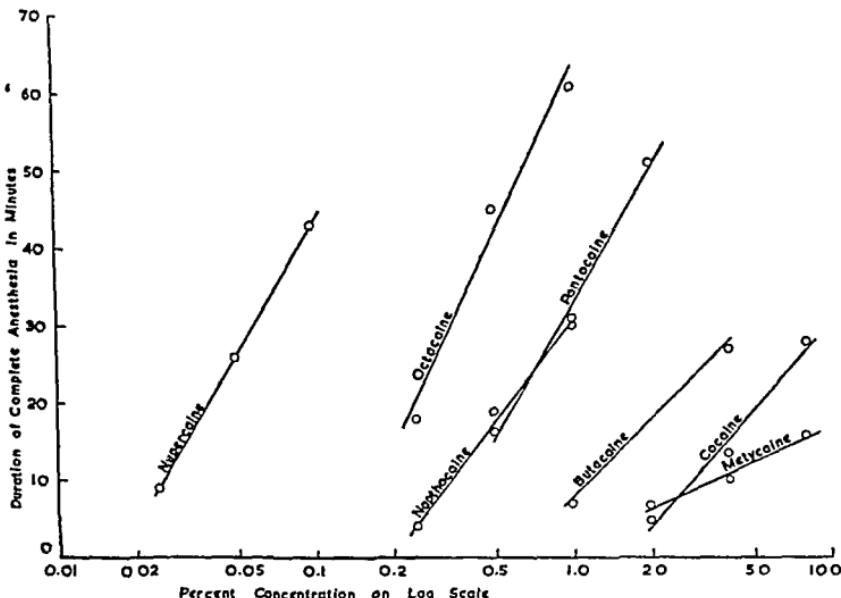


Fig. 3. DURATION OF ANESTHESIA OF CORNEA FOLLOWING INSTILLATION INTO CONJUNCTIVAL SAC OF GUINEA PIG FOR 1 MINUTE

anesthetic effects. Although the rabbit and guinea pig have both been used extensively for this purpose, Chance and Lobstein (6) state that guinea pigs are more consistent than rabbits in their corneal reflex response.

**METHOD.** Using guinea pigs weighing about 400 grams, a method similar in principle to that for infiltration anesthesia was used to evaluate surface anesthetic effects. Procaine and monocaine, having very feeble anesthetic action by topical application, were omitted from the series.

Chalk boxes, with openings for the head, were used to restrain the animals. The lashes and any other hairs around the eye that might interfere with the stimulator were clipped. The latter, following a suggestion of Hirschfelder and Bieter (7), consisted of a strip of clear celluloid about 8 cm. by 1 cm. attached at one end to a wooden handle and tapered to a point at the opposite end. A minute smooth ball of sealing wax was mounted on the point. The

indicates the concentrations at which petechiae and ulceration were observed. Although these observations are too few to allow an estimate of error, we feel that they are sufficiently regular to justify using them in computing a tentative relative rating. One observation of some interest is that the range of toxicity for skin is considerably greater than that for systemic toxicities.

**RELATIVE POTENCY (INFILTRATION).** *Method.* To assess the degree of anesthesia with different concentrations the method of Bülbürg and Wajda (5) was used, in which the wheal was pricked with a pin 6 times at intervals of 3-5 seconds. The group of 6 stimuli was repeated every 5 minutes, but instead of stopping at the end of 30 minutes the observations were continued until all stimuli evoked a response, i.e. until the anesthesia had entirely worn off. Each concentration was tested on 6 guinea pigs. The average number of failures to respond per pig was taken as the aggregate anesthetic score. From the same data the average duration of complete anesthesia was computed.

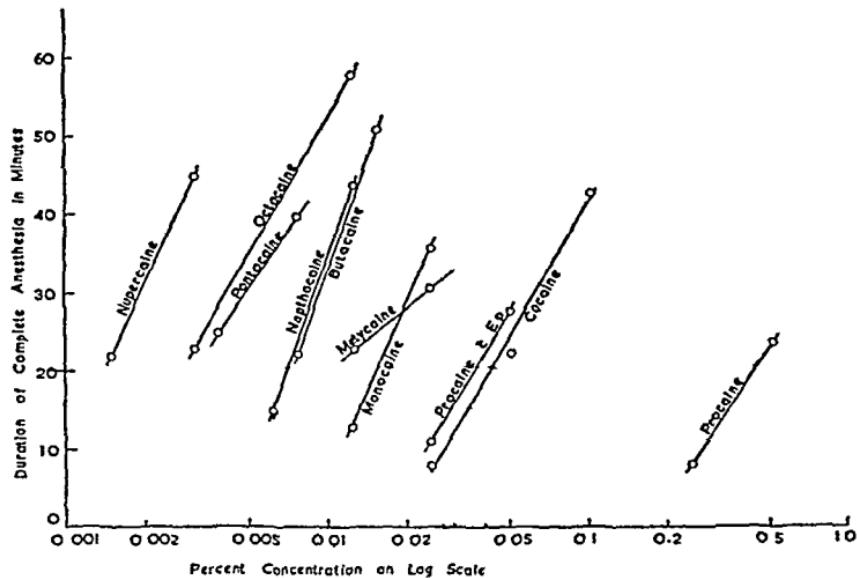


FIG. 2. DURATION OF ANESTHESIA FOLLOWING INTRADERMAL INJECTION (0.2 CC.) IN GUINEA PIGS

Each concentration of each drug except cocaine was prepared in a solution containing epinephrine hydrochloride 1:100,000. A volume of 0.2 cc. was injected intradermally into the skin of the back. Since it has been reported that the skin anteriorly is more "sensitive" than that posteriorly (5), each concentration was injected twice in an anterior, a middle and posterior position. In stimulating the wheals the pressure required was judged by that needed to make the guinea pig squeak when applied to the adjacent normal skin.

**RESULTS.** The observations were first plotted as the total number of failures to respond (aggregate anesthetic score), against the log of the concentration. These lines were approximately straight and parallel. However it seemed to us that it would be more relevant to the practical use of these drugs to examine the duration of complete anesthesia (failure of all six stimuli) as a function of log concentration. In the case of one drug, metcaine, this presentation made an

important difference which is shown in fig. 2. The slope for metycaine is definitely less than that for the others and this characteristic is also apparent in fig. 3. From the lines in fig. 2 an E.C. 50 for any duration of complete anesthesia can be read off, because each point on the line represents an average duration at that concentration. By an approximate estimation the S.E. of such an E.C. 50 is not more than 10 per cent of the value. This is applicable to all the drugs with the exception of metycaine, for which the S.E. is about 16 per cent.

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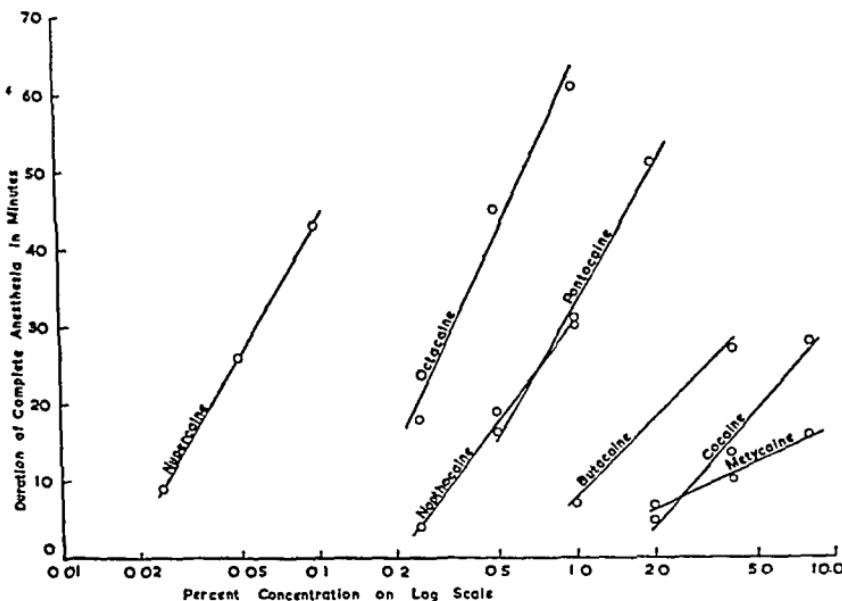


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amount of pressure exerted on the cornea by the small wax ball could be regulated by bending the celluloid any desired amount.

It was found that different degrees of pressure had to be exerted to elicit the reflex with different animals. By anesthetizing one eye only, and using the opposite eye for a control, the approximate amount of pressure necessary to induce the reflex could be gauged. The visual factor in the reflex was eliminated as far as possible by approaching the eye from the side and rear. Corneal anesthesia was produced by the instillation of 3 drops of the anesthetic, made up in distilled water, into the conjunctival sac for 1 minute, following which the eye was thoroughly flushed with normal saline. After estimating the necessary degree of pressure to cause a blink in the control eye, stimulation of the anesthetized cornea was carried out 6 times in fairly rapid succession at 5 minute intervals until anesthesia had worn off completely. Six eyes were used in this manner for each of three different percentage strengths of each drug to determine both the aggregate anesthetic score and the duration of complete anesthesia.

TABLE II  
*Relative toxicities, potencies and ratings*

DRUG	REL TOX				REL POTENCY		RELATIVE RATINGS			
	Systemic*		Tissue		Skin*	Cornea	Massive infiltration	Small n block	Eye	Mucosa
	Proc = 1	Coc = 1	Proc = 1	Coc = 1	Proc = 1	Coc = 1				
Monocaine	a	b	c	d	e	f	e/a	e/c	f/d	f/b
Procaine	0.96	0.42	0.4	0.06	2.40		2.5	6.0		
Metycaine	1.00	0.44	1.0	0.15	1.00		1.0	1.0		
Naphthocaine	1.12	0.49	5.2	0.77	2.90		2.6	0.6		
Pontocaine	1.22	0.53	3.4	0.51	5.50	9.3	4.5	1.6	18.0	13.4
Octacaine	1.80	0.79	19.4	2.88	9.00	8.8	5.0	0.5	3.0	11.3
Butacaine	1.99	0.86	19.4	2.88	13.00	21.2	6.5	0.7	7.0	24.6
Cocaine	2.01	0.88	12.7	1.88	5.30	2.3	2.6	0.4	1.0	2.6
Nupercaine	2.28	1.00	6.7	1.00	0.88	1.0	0.4	0.1	1.0	1.0
	4.03	1.76	13.7	2.04	25.90	132.0	6.0	1.9	65.0	75.0

\* For these injections all drugs except cocaine were made up with epinephrine 1:100,000.

**RESULTS.** With the exception of metycaine the lines formed by plotting percent concentration (on log scale) against duration of complete anesthesia (fig. 3) are practically parallel. When the aggregate anesthetic score was plotted against the log concentration, the line for metycaine was practically parallel to that of the other drugs.

**RELATIVE POTENCIES.** When dosage plotted against effect results in parallel lines, the relative potencies can be compared at any level of action. When they are not parallel the levels of action at which the comparison is made must be stated. In table II the relative potencies (cocaine = 1) are tabulated for a complete anesthesia for 20 minutes. The drug showing the greatest deviation from the average as regards slope is metycaine (fig. 3). It seems that partial anesthesia due to metycaine wears off more slowly than with the other drugs, which would give a misleading indication of high potency if measured by total anesthetic effect (aggregate anesthetic score) rather than by the duration of complete anesthesia.

**RELATIVE RATING INDICES.** It seems evident that for each purpose for which local anesthetics are to be used, some attempt should be made to estimate an appropriate rating index.

For massive infiltration anesthesia a relative rating based on the relative potency referred to procaine by infiltration, and the systemic toxicity related to procaine, seem most appropriate.

For conduction anesthesia of limited regions such as nerve block for dental anesthesia, the index should be based on potency relative to procaine by infiltration, and toxicity to tissues relative to procaine. It would have been better to use a relative potency figure based on conduction anesthesia, but we have been unsuccessful so far in devising a sufficiently satisfactory method for measuring potency for conduction anesthesia in mammals. Until the latter is accomplished, the R.R. (procaine) for infiltration may be of some value for nerve block but probably not for spinals because epinephrine is not used intrathecally with the anesthetic in spinals.

For surface anesthesia of the eye an index based on the potency relative to cocaine for the corneal reflex, and the relative toxicity to tissues based on cocaine, seems most relevant. For surface anesthesia of mucous cavities the potency relative to cocaine for the cornea, and systemic relative toxicity based on cocaine appears to be the most relevant index available. It would, of course, be preferable to develop a technique for measuring relative potency based on cocaine applied to a mucous surface.

From these data, then, it is possible to estimate four different Relative Rating Indices.

#### 1. Massive infiltration:

$$\text{Relative Rating (Procaine)} = \frac{\text{Relative Potency (Infiltration)}}{\text{Relative Toxicity (Systemic)}}$$

#### 2. Localized blocks:

$$\text{Relative Rating (Procaine)} = \frac{\text{Relative Potency (Infiltration)}}{\text{Relative Toxicity (Tissue)}}$$

#### 3. Surface (eye):

$$\text{Relative Rating (Cocaine)} = \frac{\text{Relative Potency (Cornea)}}{\text{Relative Toxicity (Tissue)}}$$

#### 4. Surface (mucosa):

$$\text{Relative Rating (Cocaine)} = \frac{\text{Relative Potency (Cornea)}}{\text{Relative Toxicity (Systemic)}}$$

Values for the above indices for each drug are given in table II.

The S.E. of the L.D. 50 does not in any case exceed  $\pm 8$  per cent. Consequently, the S.E. of a relative toxicity will not exceed 11 per cent. For the measurements of potency the S.E. of the E.C. 50 was estimated as not exceeding 10 per cent and consequently the S.E. of a relative potency will not exceed 14

per cent. The combination of these two errors in an index of relative rating gives an S.E. of about 19 per cent. Thus the limits of confidence of the R.R. at the level  $p = 0.05$  may be stated as R.R. multiplied by or divided by the figure 1.4 or R.R. + 40 per cent or - 30 per cent. Accordingly as far as experimental error is concerned, a difference of 60 per cent in relative ratings would be significant, but a smaller one would not be significant, assuming that such indices were obtained in experiments on the same number of animals as used in our experiments. The foregoing series of estimates applies only to the Relative Ratings based on infiltration and systemic toxicity, but it seems likely that the other indices have about the same degree of precision, although there must be some doubt about the precision of estimates of relative tissue toxicity till experiments are done on more animals.

It should be pointed out here that the confidence limits outlined above refer only to experimental conditions. In applying these indices to clinical conditions there is a degree of uncertainty which defies computation. Relative potencies and toxicities of drugs are often quite different in different species. Uncertainty of this kind can be resolved only by quantitative tests on human subjects. There is no inherent impossibility in doing estimations of relative potency of these substances on humans and it is to be hoped that such tests will be done before too long.

#### SUMMARY

1. L.D. 50 values for procaine, metycaine, monocaine, naphthocaine, butacaine, octacaine, cocaine, pontocaine and nupercaine administered intraperitoneally in white mice, decrease in magnitude in the order given. Epinephrine hydrochloride in solution with each anesthetic increases the systemic toxicity of procaine and metycaine and decreases the toxicity of pontocaine and nupercaine significantly, but has no effect on the toxicity of the 5 other drugs tested.

2. Tissue toxicity in guinea pigs, as measured by intradermal injection, does not closely parallel systemic toxicity, and the spread of tissue toxicities is considerably greater. The measurement of skin toxicity is suggested as a basis for estimating relative toxicity for nerve block in dentistry and other uses involving small volumes of local anesthetic.

3. E.C. 50 values for infiltration anesthesia have been determined in guinea pigs by intradermal injections of each anesthetic together with epinephrine hydrochloride (1:100,000).

4. E.C. 50 values for surface anesthesia of the guinea pig cornea have been determined.

5. Relative Rating Indices are defined and estimated for infiltration anesthesia, localized block anesthesia, corneal anesthesia and mucous surface anesthesia.

6. Two relatively new drugs, naphthocaine and octacaine, with high Relative Ratings for all four types of anesthesia, appear to be worthy of further clinical study.

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# BIOLOGICAL ASSAY OF THYROID ACTIVITY

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Numerous methods of estimating the potency of thyroid preparations have been suggested. None of these has led to a generally accepted assay, possibly because of their difficulty or inaccuracy. Indeed, most of the procedures have not been developed beyond the stage of qualitative analysis. Wokes (1), in his tadpole assay, is one of the few workers to describe how a quantitative comparison between two thyroid preparations may be carried out and to assign limits of error to his results. Because there is no simple, efficient method of thyroid assay, even certain points of theoretical importance have yet to be settled. For instance, great disagreement still exists concerning the relative potencies of thyroid and thyroxine (2, 3, 4) and of dextro- and levo-thyroxine (5, 6, 7). Most of the evidence indicates that at least over a wide range of normal glands, the physiological activity of thyroid is related to its iodine content (2, 4, 8, 9) so that according to the standards of the U.S.P. and B.P., thyroid preparations are assayed by chemical determinations of the iodine content. Mørch (10), on the other hand, found no relation between the iodine content and the ability of a preparation to increase the metabolic rate of mice. Thus, for both academic and pharmaceutical reasons, there is a great need for a simple, accurate bioassay of the thyroid hormone.

In this paper the results of two different methods for estimating the physiological potency of thyroxine are presented. One is based upon the decreased resistance of mice to anoxia, the other upon an early, acute weight loss in rats following the administration of thyroxine. This latter effect does not appear to have been previously described. The precision of these assays is not entirely satisfactory but they have the advantage that relatively simple procedures are involved.

**RELATION OF THYROXINE TO THE SUSCEPTIBILITY OF MICE TO ANOXIA.** In 1920, Duran (11) observed that rats treated with thyroid became more sensitive to oxygen lack. Smith, Emmens and Parkes (12) in 1947 also noted this phenomenon. They found that the administration of thyroid to mice reduced their resistance to anoxia and, thereby, decreased their survival time in closed vessels. The present study is an attempt to ascertain the precision of an assay in which groups of mice treated with various doses of thyroxine are subjected to anoxia simultaneously in a large, sealed chamber.

**METHOD.** Adult mice, weighing from 20 to 25 grams, were divided into four groups of ten animals. One group served as a control while the other three groups were given subcutaneous injections of 2.5, 5.0 and 10.0 micrograms of thyroxine daily for seven days. Forty-eight hours after the last injection, the mice were all placed together in an air-tight chamber containing soda lime. The capacity of the chamber was 32 liters. As the oxygen

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was consumed from the air, the animals became asphyxiated and died. When approximately half the mice had died, i.e. after about 45 minutes, the survivors were removed and the mortality rate of each group was noted.

**RESULTS.** In preliminary experiments, mice that were injected with only three daily doses of 0.002 to 0.250 mgms. of thyroxine were asphyxiated in a sealed chamber. The mortality rate was higher in the groups of mice treated with the larger doses of thyroxine. When the percentages of animals killed were converted to probits and plotted against the dosages of thyroxine in logarithms, a straight line was obtained. The slope of this line, however, was only 1.04. By reducing the amount of thyroxine injected and extending the doses over a period of seven days, the slope of the dose-mortality curve of mice subjected to anoxia was increased to the values shown in table I. The method of calculating the slope and its standard error was that described by Finney (13), allowances being made for the mortality rate of the control groups. The weighted mean slope was found to be 3.53 with a standard error of  $\pm 0.76$ .

**RELATION OF THYROXINE TO THE WEIGHT OF ADULT RATS.** Several workers have observed changes in the weight of animals following thyroid therapy.

TABLE I  
*Mortality rates of mice*

EXPT.	LOG DOSE THYROXINE IN MICROGRAMS				SLOPE (b)	STANDARD ERROR OF b
	Controls	0.398	0.699	1.000		
I	1/10	4/10	6/10	10/10	3.62	$\pm 1.52$
II	1/10	2/10	5/10	8/10	3.27	$\pm 1.47$
III	1/10	2/9	6/9	8/9	3.69	$\pm 1.40$

Koger, Hurst and Turner (14) found that thyroxine-treated female mice gained more weight than control mice. The results of Cameron and Carmichael (2) indicate that small doses of thyroid over a two-month period decrease the growth rate of young rats. Gaddum (5) found in adult rats that a loss of weight accompanied the rise in the metabolic rate after the administration of thyroxine.

Experiments were primarily designed to study the difference between the weight loss of thyroid-treated rats with increased metabolic rates and the weight loss of normal animals during a 24-hour starvation period. No significant difference in the amount of weight lost by the rats of the two groups was detected. During the course of these experiments, however, it was observed that the rats, without restriction of food or water, lost weight during the 48-hour period following the first injection of thyroxine. Experiments, therefore, were carried out in order to ascertain whether this loss was a significant one and to see whether it bore a relation to the dose of thyroxine.

**METHOD.** Adult male albino rats weighing 220-280 grams were given drinking water containing 0.1 per cent thiouracil for ten days prior to the injection. They were then placed in individual cages and assigned at random to the various treatments. Food (which consisted of fox chow) and water were not restricted. Two subcutaneous injections of 0.25 to

2.0 mgm. thyroxine were given to the rats on successive days. The body weights were measured at the same time every morning for several days before and after the injections.

**RESULTS.** The results of two experiments are presented in table II. These data were subjected to an analysis of variance (table III) according to the method of Bliss and Marks (15). The relationship of the weight loss of the rats to the logarithm of the dose of thyroxine may be represented by a straight line with a slope of 9.1 grams per unit log dose and a standard deviation of single obser-

TABLE II

EXPERIMENT 1				EXPERIMENT 2				
Log dose thyroxine (+1.0)								
0.398	0.699	1.000	1.301	0.398	0.699	1.000	1.301	
Weight loss of individual rats								
1	7	18	17	3	4	9	18	
6	13	12	14	4	7	17	10	
13	10	4	12	3	11	11	19	
12	6	13	24	14	8	17	12	
6	6	11	6	14	15	16	17	
2	6	15	12	8	19	14	21	
6	6	16	21	6	8	15	15	
12	16	12	16	10	16	9	15	
Mean..	7	9	13	15	8	11	14	16

TABLE III  
*Analysis of variance*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Between experiments	1	21.0
Between doses	3	200.5*
Linear response	1	599.5*
Deviations from linearity	2	1.0
Experiments x dose interaction	3	2.6
Experimental error	56	19.9
Total .	63	27.5

\* Significant at the 1 per cent level.

vations of 4.4 grams decrease in body weight. By an analysis of covariance, a slight relation was found between the initial body weight of the rats and their weight loss ( $r = +0.43$ ). The larger animals lost more weight than the smaller ones injected with the same dose of thyroxine. However, since this correlation is small, no advantage is gained by correcting for the initial weight.

An assay with a slope of 9.1 and a standard deviation of 4.4 would require 400 animals (200 on the standard and 200 on the unknown) for fiducial limits of  $\pm 25$  per cent at  $p = 0.95$ .

The acute transient weight loss is produced by thyroxine and thyroid but not by diiodotyrosine, when these substances are administered on an equivalent iodine basis (fig. 1). A significant decrease in the water and food consumption of the rats accompanies their weight loss during the 24 hours following the first thyroxine injection. Although normal rats also lose weight after the administration of thyroxine, their response is significantly less than that of animals maintained on thiouracil (fig. 2).

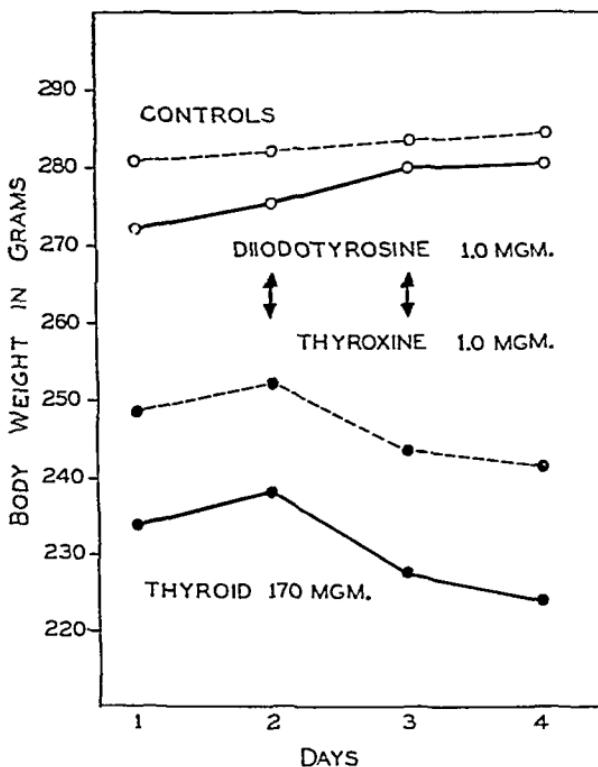


FIG. 1. CHANGES IN THE BODY WEIGHT OF RATS

Rats maintained for 10 to 14 days on thiouracil and then given two injections of diiodotyrosine, thyroxine or thyroid on an equivalent iodine basis. Each point represents the mean of the weights of 8 rats. The arrows indicate the time of the injections.

**DISCUSSION.** When the graph relating the response of an organism to the logarithm of the dose of a drug is a straight line, the ratio of the standard deviation to the slope of the line ( $s/b$ ) (16) is an estimate of the inherent precision of the assay. The greater this ratio, the more precise is the assay for a given number of animals. In table IV, several methods of measuring thyroid activity are listed. The slope of the log dose, response curve and the standard deviation have been estimated as well as possible from the published data.

Direct measurement of the metabolic rate of mice (10) has given the most precise results for determining thyroid activity. However, a simpler method

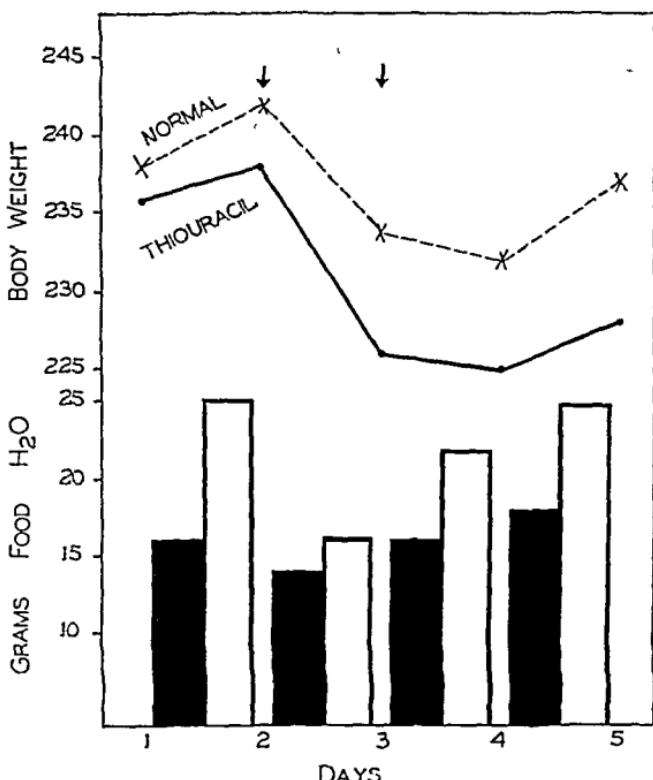


FIG. 2. CHANGES IN THE BODY WEIGHT, FOOD (SOLID COLUMNS) AND WATER CONSUMPTION (CLEAR COLUMNS) MAINTAINED ON THIOURACIL FOR 10 TO 14 DAYS AND THEN GIVEN TWO INJECTIONS OF 1.0 MG.M. THYROXINE

Changes in the weight of normal rats, not treated with thiouracil, after thyroxine injections are represented by the dotted lines. The arrows indicate the time of the injections. Each point and column represents the mean of 8 observations.

TABLE IV  
*Precision of potential assays of thyroxine and thyroid powder*

DRUG	RESPONSE	ANIMAL	STD DEV. (s)	SLOPE (b)	s/b or $\lambda$	REFERENCE
Thyroid	Decrease in length	Tadpoles	8.2	22	0.38	Wokes (1)
Thyroid . .	CO <sub>2</sub> output	Mice	2.3	46	0.05	Mørch (10)
Thyroxine.	Cardiac rates	Thyroidec-tomized rats	16	31	0.52	Leblond & Hoff (17)
Thyroxine .	CO <sub>2</sub> output	Rats on thiouracil	28	81	0.35	Reinecke, Mixner & Turner (18)
Thyroxine	Thyroid gland size	Rats on thiouracil	1.7	11	0.16	<i>Ibid.</i>
Thyroxine	Weight loss	Rats on thiouracil	4.4	9.1	0.48	
Thyroid . . .	Basal metabolic rate	Man	7.0	38	0.18	Winkler, Criscuolo & Lavietes (3)

than that described by Mørch is desirable for routine assays. Standardization of thyroid by measuring the weight loss of rats is easily performed in relatively short time but a very large number of animals is necessary for this method to be satisfactory. When allowance is made for the fact the asphyxiation test is a quantal response, the accuracy of the two methods is approximately the same. Nevertheless, compared to the values listed by Bliss and Cattell (16), the precision of the asphyxiation test is of the same order as that of other assays based upon the all-or-none response. This test has the advantage that it involves a very simple technique although it may not be specific for the thyroid hormone.

The weight loss following the injection of thyroxine was quite unexpected. The decrease in body weight reported by Gaddum (5) was later in onset than that found in our experiments and occurred in rats kept on a restricted food intake, whereas our animals ate and drank as they pleased. The weight loss observed by Gaddum may well have reflected the rise in metabolic rate. In the experiments reported here, it appears to be the result of a voluntary decrease in water consumption. The problem deserves further investigation.

#### SUMMARY

Two methods to measure thyroid activity are described and compared with other bioassays of this hormone.

One is a quantal response type of assay depending upon the decreased resistance to anoxia of mice treated with thyroxine. The slope (*b*) for this procedure was  $3.53 \pm 0.76$ .

The other is a graded response method based on the acute weight loss of rats during the 48 hours following the first of two injections of thyroxine. The standard deviation of this method was 4.4 and the slope of the log dose response curve was 9.1. The value for  $\lambda$  was, therefore, 0.478.

The acute, transient loss of weight is apparently due to a reduction of the water and food intake by the animals. It is not produced by injections of diiodotyrosine. Rats previously treated with thiouracil have a greater transient weight loss after the administration of thyroxine than have normal rats.

**ACKNOWLEDGEMENT.** The author takes pleasure in expressing his thanks to Dr. J. K. W. Ferguson for his suggestions, advice and interest throughout this investigation. His thanks are also due to Mr. D. B. W. Reid for reviewing the statistics of the data.

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## ANESTHESIA

### XXXI. A STUDY OF CYCLIC AND NONCYCLIC HYDROCARBONS ON CARDIAC AUTOMATICITY<sup>1</sup>

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Meek, Hathaway and Orth (1) showed that arrhythmias frequently occurred in the dog's heart under cyclopropane anesthesia following intravenous injections of epinephrine. Evans, Oster and Krantz (2) extended this observation to the *Macacus rhesus* monkey. Recently Krantz et al. (3) showed that the myocardium of the dog under cyclobutane anesthesia was sensitized to epinephrine.

We became interested in extending these studies to other hydrocarbons. It was believed that such a study might shed light on the types of structures responsible for the cardiac sensitivity.

**EXPERIMENTAL PROCEDURE.** Electrocardiograms, Lead II, were recorded from the unanesthetized dogs. Epinephrine hydrochloride solution, 1:100,000 (preserved with sodium bisulfite) was injected intravenously, 0.01 mgm./kgm. The injection was made during a period of 25 to 40 seconds. At the end of the injection (usually the beginning of the effect) another tracing was made.

Each animal subsequently was permitted to breathe a mixture of the hydrocarbon in varying concentrations mixed with oxygen. With the liquid hydrocarbons the concentrations were between 10 and 25 per cent. With the gaseous compounds concentrations from 15 to 90 per cent were used. The closed circuit procedure was employed. When the inhalation had continued for approximately 10 minutes the foregoing experimental procedures were again carried out.

**OBSERVATIONS.** In table 1 the observations made with various hydrocarbons are listed.

**CARDIAC AUTOMATICITY.** The hydrocarbons in this series, with the exception of ethylene, sensitized the myocardium to epinephrine. In the dog, prior to anesthesia, epinephrine produced slowing of the cardiac rate, accentuation of the T-wave and an occasional inversion of the QRS complex. Under the influence of the hydrocarbons, however, multifocal ventricular tachycardia occurred in nearly all experiments with epinephrine. In some animals this passed into ventricular fibrillation and caused death.

Under ethylene anesthesia in 12 dogs we observed the control response of the heart to epinephrine. In this series ethylene alone appears to be unique in this respect. We attempted repeatedly to induce myocardial sensitization to epinephrine by increasing the concentration of ethylene inhaled and prolonging the exposure to the hydrocarbon. These efforts were unsuccessful. However, exposure to ethane produced cardiac sensitization in 2 of 4 animals.

<sup>1</sup> The expense of this investigation was defrayed in part by a grant from The Ohio Chemical & Mfg. Co., Cleveland, Ohio.

To obviate the factor of animal variation epinephrine was injected into a dog under ethylene anesthesia and two hours later the same dog was treated with epinephrine under cyclopropane anesthesia. The results are shown in figure 1.

In these studies 12 dogs were used with ethylene and 2 to 5 with each of the other agents. Ventricular fibrillation occurred in 5 of 20 dogs in Orth's (5) series with cyclopropane. In our series of 34 dogs, exclusive of those used with ethylene, ventricular fibrillation occurred 19 times after epinephrine injections. Fibrillation occurred in each dog under isobutane, *cis-trans* butene-2, propylene and cyclobutene, respectively.

**CHARACTER OF THE ANESTHESIA.** Stroughton and Lamson (6) studied the anesthetic properties of butane and isobutane. They reported the agents unsatisfactory based upon their observations in dogs. We confirmed these findings. In this series of hydrocarbons only cyclobutane and cyclobutene produce a satisfactory anesthetic syndrome.

**DISCUSSION.** Stutzman et al. (7) produced evidence to show that cyclopropane sensitizes the heart of the dog reflexly to epinephrine. The receptors

TABLE 1

NAME OF COMPOUND	NUMBER DOGS IN WHICH SENSITIZATION OF MYOCARDIUM OCCURRED AND NUMBER USED	NAME OF COMPOUND	NUMBER DOGS IN WHICH SENSITIZATION OF MYOCARDIUM OCCURRED AND NUMBER USED
Ethane	2/4	<i>Cis-trans</i> -butene-2	2/2
Ethylene	0/12	Cyclobutene	5/5
Propane	3/3	Cyclobutane	2/2
Propylene	2/2	Cyclopentane	2/3
n-Butane	2/2	Isopentane	3/3
Isobutane	2/2	2,2-Dimethyl-butane.	3/3

are distributed mainly throughout the peripheral 3 cm. of the mesentery. Nerve impulses pass by visceral afferent fibers through the celiac and superior mesenteric plexuses, splanchnics and spinal cord to a brain center above the pons. The efferent impulses then reach the heart by way of the cardiac sympathetics and thus the irritability of the myocardium is increased.

It is striking that the sensitization is evoked by this entire series of hydrocarbons but not by ethylene. Levy (8) recognized in 1912 that certain anesthetic agents, particularly chloroform, might sensitize the heart to stimuli of various kinds so that tachycardia and fibrillation might result.

We have observed that chloroform occasionally sensitizes the myocardium of the dog to epinephrine. Likewise Oster, Carr and Krantz (9) showed that anesthesia with vinyl chloride produced abnormalities in the QRS complex varying from sinus arrhythmia to ventricular tachycardia, deep downward prolongation of the S-wave and ventricular fibrillation in the dog. In contrast to the anesthesia with these hydrocarbons and certain of their halogenated derivatives, anesthesia with 8 different symmetrical and unsymmetrical ethers did not produce cardiac arrhythmias or sensitization (10).

Chenoweth (4) studied the sensitization of the myocardium to epinephrine when animals were exposed to vapors of various hydrocarbons including gasoline.

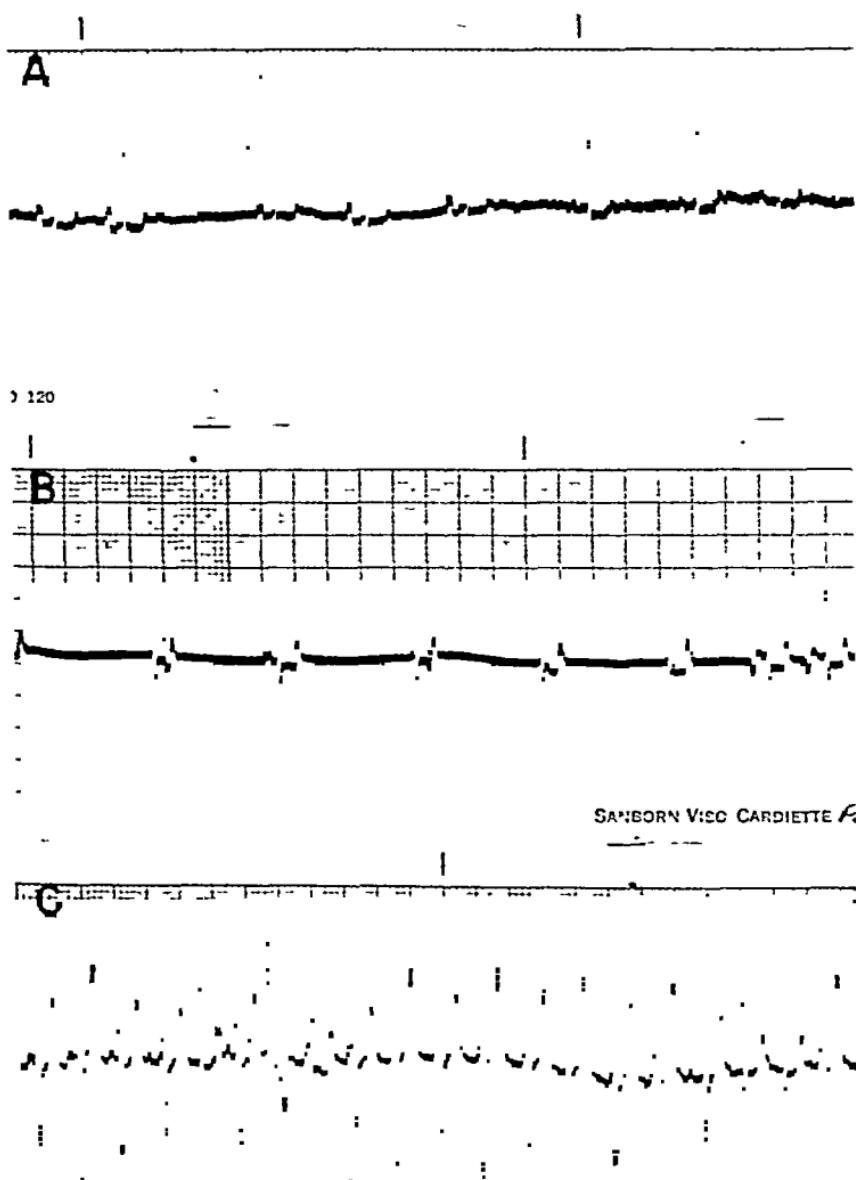


FIG 1 a Electrocardiogram of dog, Lead II, after epinephrine b Same as "a" under ethylene anesthesia c Same as "a" under cyclopropane anesthesia

In this series he observed that along with other aliphatic and aromatic hydrocarbons, methane and n-butane caused sensitization of the heart. Later Garb

and Chenoweth (11) suggested from their studies on the isolated heart and on papillary muscle preparations that ventricular fibrillation is initiated when an area of myocardium containing a slightly higher concentration of hydrocarbon than the remainder of the heart fails to respond to a QRS stimulus, but does respond to the following T-wave.

Convulsive seizures are apparently not a factor since the arrhythmias are produced as readily under cyclobutene which evokes deep anesthesia and excellent relaxation as under *cis-trans* butene-2, which produces a stormy anesthetic syndrome.

#### CONCLUSIONS

1. The inhalation of ethylene does not sensitize the dog's myocardium to epinephrine.
2. The inhalation of 12 other hydrocarbons reported in this paper produces sensitization.
3. Cyclobutane and cyclobutene produce excellent anesthetic syndromes in the dog. The other hydrocarbons studied are unsatisfactory anesthetic agents in the dog.

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# THE ROLE OF ARTERIAL PRESSURE IN THE INDUCTION OF IDIOVENTRICULAR RHYTHMS UNDER CYCLOPROPANE ANESTHESIA<sup>1</sup>

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The production of ventricular premature contractions, ventricular tachycardia, and ventricular fibrillation by epinephrine during exposure to chloroform, benzol, cyclopropane, and other agents has been frequently demonstrated (1, 2). The mechanism is not yet understood; it has recently been shown that hydrocarbons which are capable of "sensitizing" the heart to epinephrine do not increase the excitability of isolated cardiac tissue, but may actually increase the threshold for electrical stimulation (3). The elevation of pressure produced by epinephrine has been considered to play a prominent role, since Rothberger and Winterberg (4) have shown that a mechanically induced pressure rise (aortic occlusion) may cause the appearance of ventricular premature contractions and a bigeminal rhythm, particularly if vagal inhibition of the sinus node is not prevented. Ventricular irregularities resulting from large doses of epinephrine in rabbits under barbital anesthesia can be prevented by use of a pressure stabilizer (5). Shen (6), postulating that the rise of arterial pressure produced by epinephrine was responsible for the ventricular arrhythmias induced in the heart sensitized by chloroform, studied the effect of 933 F and yohimbine, agents which inhibit or reverse the pressor effect of epinephrine. In accordance with his theory, prevention of the pressure rise protected the heart against epinephrine-chloroform fibrillation. The possibility that the protective action of these agents was related to a direct antagonism of the cardiac actions of epinephrine was, however, not excluded. Allen, Stutzman, and Meek (7) found that ergotamine in doses too small to prevent the pressor response to epinephrine also afforded protection against idioventricular rhythms. They found that decerebration, cord section, or cardiac sympathetic denervation, which did not prevent the pressor response, prevented ventricular tachycardia or greatly reduced its duration even after several times the dose of epinephrine which was effective in control trials. They concluded that sympathetic impulses to the heart are important for the production of ventricular tachycardia, and that the action of cyclopropane might be explained at least in part by a central or reflex activation of sympathetic mechanisms. Recently these workers have postulated a reflex mechanism whose afferent limb originates in the mesentery or intestine (8).

Dibenamine also protects the heart against the cardiotoxic effects of cyclopropane and epinephrine (9). Although Dibenamine reverses the pressor action

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of epinephrine, Acheson, Farah, and French (10) found that it does not interfere with the positive chronotropic and inotropic effects of epinephrine on the dog heart. Since Dibenamine does not block these specific cardiac actions of epinephrine, it appeared likely that the protection against ectopic rhythms might be related to its ability to prevent the pressor response. The following series of experiments were planned to determine the validity of this hypothesis, and also to assay the general importance of arterial pressure in the induction of idioventricular activity with epinephrine.

**METHODS.** Dogs ranging in weight from 8 kgm. to 16 kgm. were used in 40 acute experiments. Anesthesia was on occasion induced and maintained throughout with 20 to 33 per cent cyclopropane in oxygen administered from a clinical anesthesia apparatus; more frequently narcosis was induced with pentothal, and the necessary operative procedures were carried out before offering the cyclopropane-oxygen mixture. The closed system was flushed out frequently with the gas mixture in order to maintain a reasonably constant anesthetic concentration.

Blood pressure was recorded with a mercury manometer attached to a carotid artery. The electrocardiograms were taken with leads clipped into the subcutaneous tissue of the neck and the left groin, using a "Cardiotron" direct-writing instrument.

In some experiments the chest was opened and the aorta exposed for intermittent clamping. In others a pressure-regulator was attached to the abdominal aorta. The pressure-regulator was constructed from a one-liter graduated cylinder, enclosed in a glass jacket through which water at 38°C. was circulated. The air in the cylinder communicated with a mercury manometer and with two large carboys of a total volume of 36 liters. The blood in the chamber communicated with the abdominal aorta through two cannulas: one from the bottom of the vessel was directed toward the heart; the other, opening about 10 cm. above the bottom of the vessel, was directed peripherally, thus permitting circulation to the legs and also insuring continuous replacement of blood in the reservoir. To prevent clotting heparin was administered by continuous infusion, and surfaces exposed to blood were coated with Dow Corning "200 Fluid" silicone. The regulator could be clamped off at will. When open, the instrument compensated for depressor or pressor reactions in the animal by infusing or taking up blood. In some experiments the regulator was used to produce a sudden increase or decrease of aortic pressure; in others it was used to set the aortic pressure at various levels in order to compare the responses to epinephrine at different pressures.

Heart-lung preparations were performed according to standard procedures on dogs anesthetized with sodium pentobarbital. In these experiments a fixed resistance (screw-clamp) was frequently substituted for the customary Starling resistance. Cyclopropane was introduced as desired in concentrations of 33 to 50 per cent.

Epinephrine was always injected rapidly; the method recommended by Meek, of injecting 0.01 mgm./kgm. over a period of 50 seconds, could not be used, since it was necessary to determine a threshold dose, and since it was desired to produce ventricular tachycardia several times in the same experiment. To eliminate the danger of ventricular fibrillation, the initial dose was small, usually 0.5 microgram/kgm. or less; increasing doses were then injected until tachycardia was produced.

**RESULTS.** *The action of dibenamine and  $\alpha$ -naphthylmethylethyl- $\beta$ -bromoethylamine (SY-28).* We have been able to confirm the results of Nickerson and Smith (9) and of Acheson, Farah, and French (10) that Dibenamine in doses of 15 to 20 mgm./kgm. regularly protects the dog heart against epinephrine and cyclopropane. We have made no attempt to determine quantitative relationships, but doses of epinephrine which repeatedly caused ventricular tachycardia

in the control period always failed to do so after Dibenamine, and doses 2 to 4 times the initial test dose were tolerated without arrhythmia. The doses of Dibenamine which were used converted the pressor action of epinephrine to a predominantly depressor effect.

Alpha-naphthylmethylethyl- $\beta$ -bromoethylamine, a more potent homologue of Dibenamine (11), also reverses the pressor action of epinephrine and protects the heart against ventricular tachycardia in doses of 1 mgm./kgm. The data below apply to both drugs.

In order to demonstrate that the protective action of these drugs is due to their ability to "reverse" the blood pressure response to epinephrine, experiments were conducted with the following order of procedure: (1) control threshold dose of epinephrine causing tachycardia (2) injection of the anti-epinephrine drug (3)

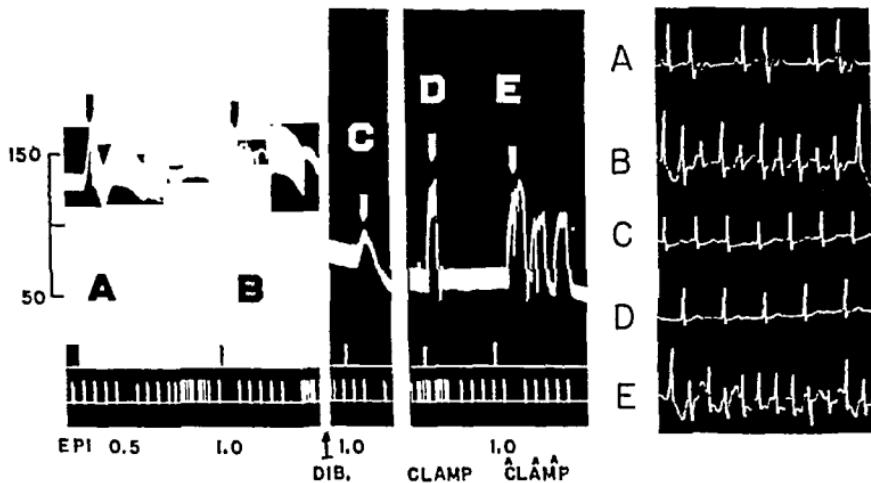


FIG. 1. EXP. 8-27-47. DOG 12 KGM. CYCLOPROPANE-OXYGEN, ARTIFICIAL RESPIRATION, CHEST OPEN

Lifting ligature under aortic arch. Tracings from top to bottom; arterial pressure (scale at left), signal, time in 10 sec. Arrows indicate time at which ECG records taken. Epi = epinephrine, dose in micrograms per kgm. Between B and C, Dibenamine 15 mgm./kgm. followed by 30 minute interval. At "clamp", aorta occluded. ECG segments, 3 seconds

repetition of the control test dose of epinephrine, or larger doses (4) temporary mechanical elevation of pressure, either by compression of the aorta or by elevation of the pressure in the regulator and (5) injection of the test dose of epinephrine followed within 5 seconds by mechanical elevation of the pressure.

Typical results are illustrated in fig. 1. Epinephrine, 0.5 microgram/kgm., caused a bigeminal rhythm; twice this dose caused a multifocal tachycardia. After Dibenamine, epinephrine failed to disturb the normal sinus rhythm; elevation of pressure alone (induced by aortic clamping) was also without effect; but aortic clamping shortly after the injection of epinephrine produced the characteristic ventricular rhythm. Normal rhythm was restored by releasing the clamp, and tachycardia was again produced by a second brief period of aortic occlusion.

Similar results were obtained when the elevation of pressure was accomplished with the aid of the pressure-regulator. In such experiments it was evident that a sudden rise of pressure is not necessary for the production of ectopic rhythms. For example, in one experiment, 1 microgram/kgm. of epinephrine produced ventricular tachycardia in the control period, while 8 micrograms/kgm. failed to do so after 1 mgm./kgm. of SY-28. The pressure was then set above the level at which the tachycardia occurred with the control test dose of epinephrine and the regulator was kept open. Epinephrine, 1 microgram/kgm., again-caused ventricular tachycardia without any further significant change of pressure. As in the experiments with aortic occlusion, tachycardia once produced could always be terminated by reducing the pressure.

The protective action of Dibenamine was demonstrated twenty-four hours after administration of the drug in one experiment; mechanical elevation of the pressure restored the characteristic arrhythmic response to epinephrine as in acute experiments.

*The relation of pressure to effective dose of epinephrine.* Since it is evident that Dibenamine protects the heart at least in part because it prevents the pressor action of epinephrine, it must follow that any other means of preventing the pressor response would also prevent the ectopic rhythms. This hypothesis was tested in experiments with the pressure-regulator. Responses to epinephrine were studied during control trials with the regulator excluded and compared with the responses obtained with the regulator open. Epinephrine was injected in ascending doses until ventricular tachycardia of significant duration was produced (doses ranging from 0.5 to 4.0 micrograms/kgm.). The pressure regulator was then opened at the prevailing pressure of the dog, and the effective test dose of epinephrine was repeated. Arterial pressure rose no more than 5 or 10 mm. Hg, and ventricular tachycardia was never observed. Epinephrine was then injected in increasing doses (increments of 100 per cent); in one experiment ventricular tachycardia occurred with 4 times the dose effective in the control period; in others as much as 16 micrograms/kgm. (8 times the control dose) were tolerated without ectopic rhythm. At intervals the initial test dose was repeated with the regulator excluded in order to be sure there was no progressive change in the sensitivity of the heart.

Figure 2 illustrates a typical experiment. Ascending doses of epinephrine were injected with the pressure-regulator excluded; ventricular tachycardia was produced by 4 micrograms/kgm. (part A). With the pressure-regulator open, 16 micrograms/kgm. failed to alter arterial pressure significantly and also failed to disturb the sinus rhythm (part B). The test dose of 4 micrograms was repeated in C and again, after atropinization, in part D, with resultant ventricular tachycardia in each case.

Preventing a pressor response to epinephrine does not offer complete protection, as illustrated in figure 3. In this dog, 1 microgram/kgm. did not disturb the normal rhythm, but 2 micrograms/kgm. caused ventricular tachycardia with the pressure-regulator closed. With the regulator open at 100 mm. Hg, and again at 120 mm., the latter dose failed to cause any disturbance of rhythm, but

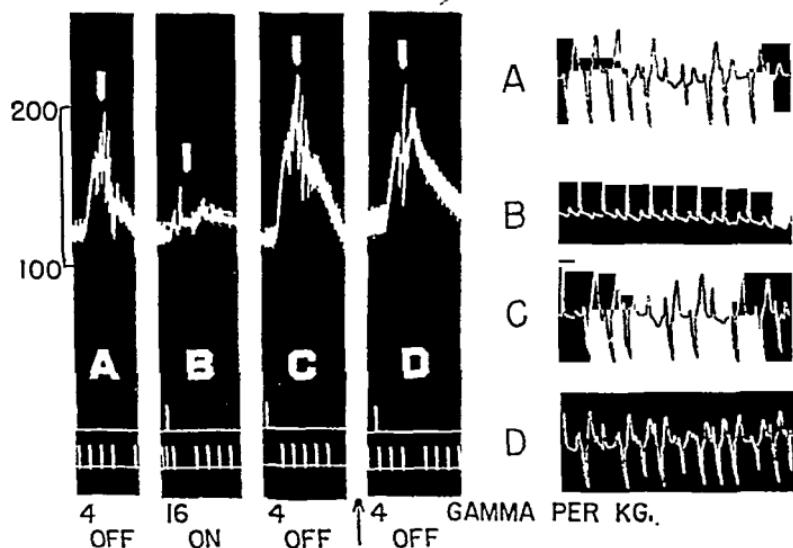


FIG. 2. EXP. 9-12-47. DOG 15 KGM. CYCLOPROPANE-OXYGEN

Pressure regulator in abdominal aorta. Tracings as in figure 1. Figures indicate dosage of epinephrine. "Off" and "on" indicate pressure regulator closed or open. ECG segments, 3 seconds.

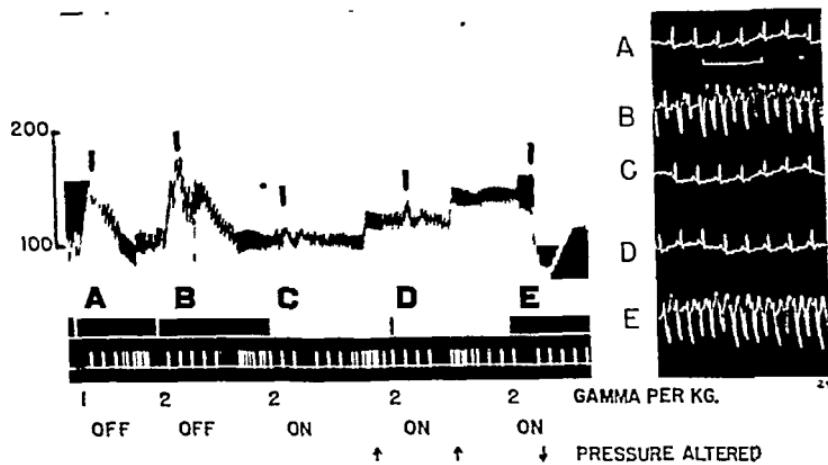


FIG. 3. EXP. 9-24-47. DOG 12.3 KGM. CYCLOPROPANE-OXYGEN

Pressure regulator in abdominal aorta. Tracings as in figure 1. Figures at bottom; dosage of epinephrine. "Off" and "on", pressure regulator closed or open. Alterations of pressure accomplished by means of pressure regulator. Bracket in ECG segment A = 1 sec.

at 140 mm., the same dose caused a severe ventricular tachycardia which was terminated by dropping the pressure in the system.

The relationship between pressure and threshold dose is illustrated in figure 4. The responses of this animal were particularly stable; it was possible to return to

the original pressure and effective dose several times during a rather long experiment. Pressure was altered above or below the original level, without any orderly sequence, and the threshold dose determined at each setting. In the range between 80 and 160 mm. Hg, the threshold dose of epinephrine was reduced by half with each 20 mm. elevation of pressure.

It is obvious from these experiments that the level of arterial pressure is one of the important factors determining the sensitivity of the heart to epinephrine.

*The role of reflex mechanisms.* When epinephrine causes an elevation of arterial pressure in the intact animal, carotid and aortic reflexes will increase vagal tone and reduce sympathetic tone to the heart. Ventricular premature contractions produced by clamping the aorta will often fail to appear if the vagi are cut, since the sinus node then retains control over the ventricle. It seems

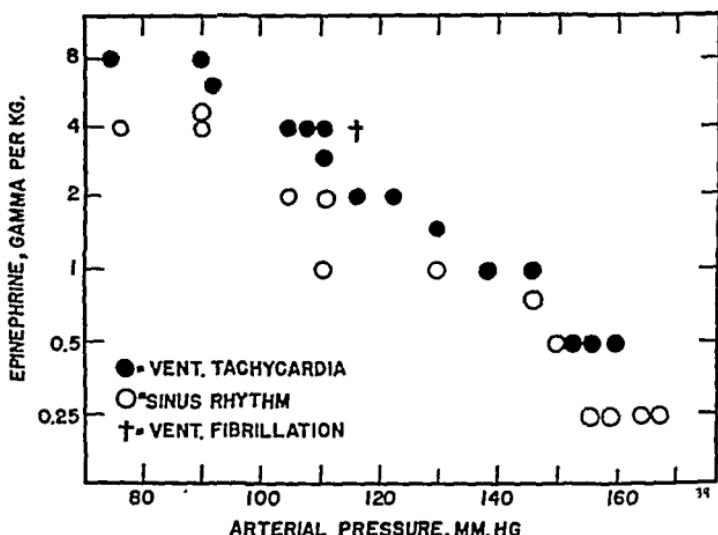


FIG. 4. EXP. 10-21-47. DOG 8 KG.W. CYCLOPROPANE-OXYGEN

Pressure regulator in abdominal aorta. Relation between dose of epinephrine and arterial pressure necessary to produce ventricular tachycardia.

possible, then, that prevention of the pressor response to epinephrine might protect the heart by preventing such cardiodecelerator reflexes. In several experiments atropine or tetraethylammonium or both were administered in order to test the importance of reflex compensatory mechanisms.

Atropine in doses up to 0.5 mgm./kgm. failed to protect the heart, as illustrated in the last segment of figure 2. This is in accord with previous observations. Tetraethylammonium, which would be expected to block both vagal and sympathetic impulses to the heart, also failed to alter significantly the threshold dose of epinephrine. A typical result is illustrated in figure 5. In this experiment 1.0 microgram/kgm. caused sinus fibrillation, 1.5 caused ventricular fibrillation, and 1.5 caused ventricular tachycardia during the infusion of tetraethylammonium. During the infusion of tetraethylammonium, the same doses of epinephrine. Ergotamine, however, in a dose of 0.05 mgm./kgm. protected the heart against 4 times the previous threshold dose of epinephrine.

ously effective dose of epinephrine, even though it did not prevent a pressor response.

It is apparent, then, that the effect of pressure is exerted on the myocardium directly; reflex adjustments do not play an essential role. It is further evident that ergotamine, which can protect the heart in doses which cause no "epinephrine reversal," may interfere with the direct myocardial action of epinephrine.

*Ventricular arrhythmias in the heart-lung preparation.* Since functional "denervation" of the heart with the aid of tetraethylammonium does not protect against ventricular rhythms, it should be possible to produce this characteristic response to epinephrine and cyclopropane in the isolated heart. The following points were studied in four dog heart-lung preparations: (1) the effect of elevating arterial pressure in the absence of cyclopropane and epinephrine (2) the effect of

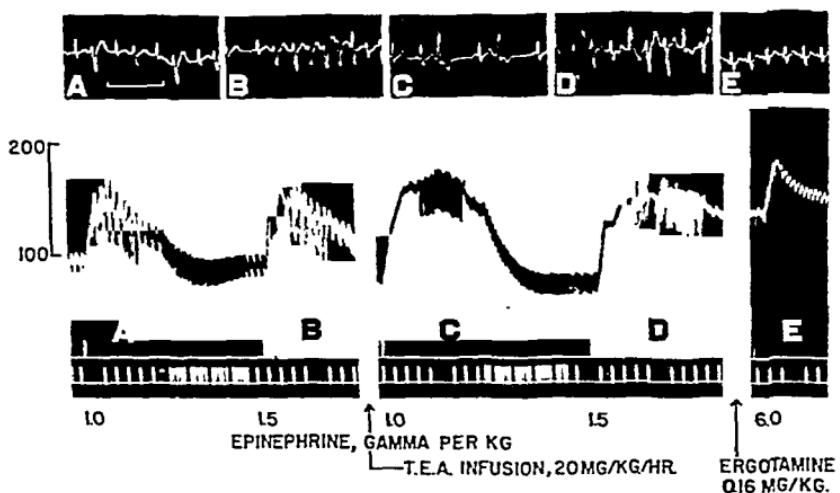


FIG. 5. EXP. 10-S-47. DOG 15.2 KGM. TRACINGS AS IN FIGURE 1. BRACKET IN ECG SEGMENT  
A = 1 SECOND

pressure elevation in the presence of epinephrine but without cyclopropane (3) the effect of pressure elevation with cyclopropane, and (4) the effect of epinephrine with or without a rise of pressure, in the presence of cyclopropane.

In confirmation of Rothberger and Winterberg (4) it was found that a sudden elevation of pressure may provoke a bigeminal rhythm in the untreated heart; gradual or step-wise elevation to even greater levels was usually without such action. When pressure was suddenly raised to high levels immediately after the injection of epinephrine (0.02 mgm. total), no ventricular premature contractions resulted, probably because the accelerated sinus node retained control and prevented the appearance of ectopic ventricular activity. One may conclude that the combination of reasonable doses of epinephrine and feasible levels of arterial pressure will not disorganize cardiac rhythms; some contributory action by the hydrocarbon is essential for the appearance of ectopic foci.

In the presence of cyclopropane, elevation of pressure alone caused only

the original pressure and effective dose several times during a rather long experiment. Pressure was altered above or below the original level, without any orderly sequence, and the threshold dose determined at each setting. In the range between 80 and 160 mm. Hg, the threshold dose of epinephrine was reduced by half with each 20 mm. elevation of pressure.

It is obvious from these experiments that the level of arterial pressure is one of the important factors determining the sensitivity of the heart to epinephrine.

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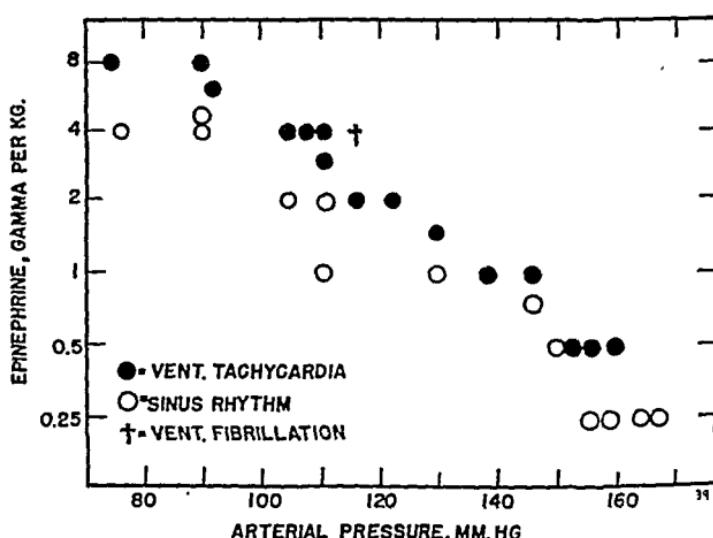


FIG. 4. EXP. 10-21-47. DOG 8 KGM. CYCLOPROPANE-OXYGEN

Pressure regulator in abdominal aorta. Relation between dose of epinephrine and arterial pressure necessary to produce ventricular tachycardia.

possible, then, that prevention of the pressor response to epinephrine might protect the heart by preventing such cardiodecelerator reflexes. In several experiments atropine or tetraethylammonium or both were administered in order to test the importance of reflex compensatory mechanisms.

Atropine in doses up to 0.5 mgm./kgm. failed to protect the heart, as illustrated in the last segment of figure 2. This is in accord with previous observations. Tetraethylammonium, which would be expected to block both vagal and sympathetic impulses to the heart, also failed to alter significantly the threshold dose of epinephrine. A typical result is illustrated in figure 5. In this experiment 1.0 microgram/kgm. caused a bigeminal rhythm, and 1.5 caused ventricular tachycardia during the control period. During the infusion of tetraethylammonium, the same responses followed the same doses of epinephrine. Ergotamine, however, in a dose of 0.16 mgm./kgm., protected the heart against 4 times the previ-

ously effective dose of epinephrine, even though it did not prevent a pressor response.

It is apparent, then, that the effect of pressure is exerted on the myocardium directly; reflex adjustments do not play an essential role. It is further evident that ergotamine, which can protect the heart in doses which cause no "epinephrine reversal," may interfere with the direct myocardial action of epinephrine.

*Ventricular arrhythmias in the heart-lung preparation.* Since functional "denervation" of the heart with the aid of tetraethylammonium does not protect against ventricular rhythms, it should be possible to produce this characteristic response to epinephrine and cyclopropane in the isolated heart. The following points were studied in four dog heart-lung preparations: (1) the effect of elevating arterial pressure in the absence of cyclopropane and epinephrine (2) the effect of

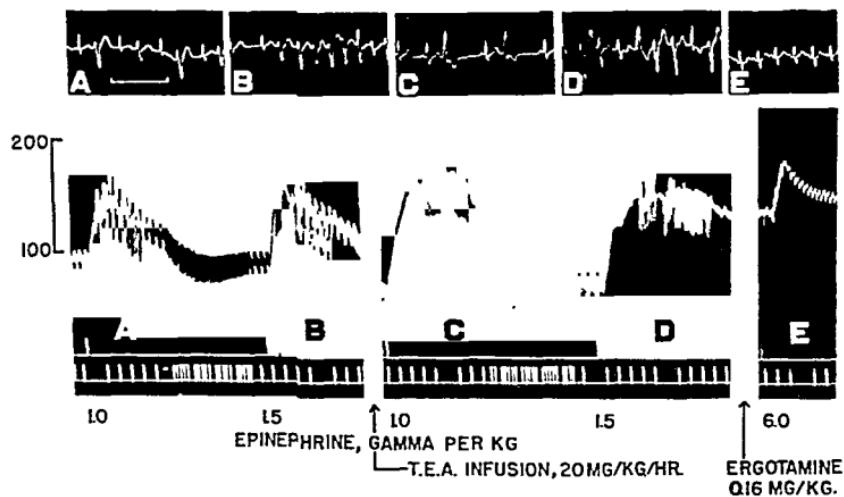


FIG. 5. EXP. 10-S-47. DOG 15.2 KGM. TRACINGS AS IN FIGURE 1. BRACKET IN ECG SEGMENT A = 1 SECOND

pressure elevation in the presence of epinephrine but without cyclopropane (3) the effect of pressure elevation with cyclopropane, and (4) the effect of epinephrine with or without a rise of pressure, in the presence of cyclopropane.

In confirmation of Rothberger and Winterberg (4) it was found that a sudden elevation of pressure may provoke a bigeminal rhythm in the untreated heart; gradual or step-wise elevation to even greater levels was usually without such action. When pressure was suddenly raised to high levels immediately after the injection of epinephrine (0.02 mgm. total), no ventricular premature contractions resulted, probably because the accelerated sinus node retained control and prevented the appearance of ectopic ventricular activity. One may conclude that the combination of reasonable doses of epinephrine and feasible levels of arterial pressure will not disorganize cardiac rhythms; some contributory action by the hydrocarbon is essential for the appearance of ectopic foci.

In the presence of cyclopropane, elevation of pressure alone caused only

occasional ventricular premature contractions, but in contrast to experiments without cyclopropane, a pressure rise produced a few seconds after the injection of small doses of epinephrine produced multifocal ventricular premature contractions or frank ventricular tachycardia. By substituting a screw clamp for the customary Starling resistance and inducing a moderate degree of failure, epinephrine can be made to produce a great elevation of arterial pressure, by virtue of a great increase of cardiac output ejected against the fixed resistance. When the Starling resistance is used, the change of arterial pressure is, of course, minimal. In one such experiment epinephrine in a total dose of 0.06 mgm. failed to cause ventricular irregularities with the Starling resistance in place, while 0.02 mgm. injected after the substitution of the fixed resistance caused a prolonged elevation of pressure and a long period of ventricular tachycardia.

While it is not possible to compare directly the effective doses of epinephrine in the heart-lung preparation with those used in the intact animal, it is probable that a total dose of 0.02 mgm. in the former is roughly comparable to the same dose in the latter, in spite of the restricted mass of tissue and blood in the isolated organ; for a dose quickly injected into the venous blood stream will reach the heart in a concentration which depends upon the speed of injection and the cardiac output, not upon the total mass of tissue in the system. It is apparent, then, that the isolated heart, separated from any possible reflex phenomena, retains a sensitivity to the combination of epinephrine, cyclopropane, and pressure.

**DISCUSSION.** It is postulated by Meek and co-workers that ventricular fibrillation occurring under cyclopropane is due in part to reflex activation of the sympathetic innervation of the heart, the afferent limb of the reflex originating apparently in the mesentery or intestine (8). Interruption of the afferent pathways, surgical excision of thoracic sympathetic ganglia, or administration of ergotamine protect the heart. Interruption of autonomic pathways by means of tetraethylammonium does not in our experience alter the threshold dose of epinephrine significantly; it is possible, then, that there are efferent nerves to the heart involved in this mechanism which can be interrupted by surgical denervation, but not by infusion of tetraethylammonium; for example, nonganglionated paths. There is no evidence for such a hypothesis, and none against it.

Ectopic rhythms, however, can also be produced by epinephrine in the heart-lung preparation exposed to cyclopropane. While there may be a quantitative difference in the dosage required, yet it is apparent that a direct action exists. It must be admitted that we have not produced ventricular *fibrillation* in such an experiment; the possibility remains that an intact reflex pathway is essential for the lethal arrhythmia to occur.

Ergotamine protects the heart in doses which do not reverse the pressor action of epinephrine; this observation is confirmed by our own experiments. Dibenamine protects against ventricular fibrillation, but not ventricular tachycardia, in cats exposed to epinephrine and various hydrocarbons (3), even in doses which fail to alter the pressor action of epinephrine. In our experiments we have shown that protection against tachycardia is related to the ability of Dibenamine

to "reverse" the pressor action of epinephrine, but again it must be admitted that we have *not* produced fibrillation in dogs so protected.

An explanation harmonious with the results of other laboratories is possible: Dibenamine protects against ventricular tachycardia by virtue of its ability to prevent the pressor response to epinephrine, but in addition, and in spite of its apparent lack of antagonism to the cardiac actions of epinephrine, it may be capable of preventing the development of fibrillation by virtue of its quinidine-like action (10), or some as yet unknown action upon the heart.

#### SUMMARY

1. Dibenamine and  $\alpha$ -naphthylmethylethyl- $\beta$ -bromoethylamine reverse the pressor action of epinephrine in dogs and protect against the induction of idioventricular rhythms under cyclopropane.
2. Idioventricular rhythms (but possibly not ventricular fibrillation) can still be induced by epinephrine after Dibenamine if arterial pressure is mechanically elevated.
3. Prevention of a pressor response to epinephrine by means of a pressure regulator also protects markedly, but not completely, against the appearance of idioventricular activity.
4. There is a quantitative relationship between the level of arterial pressure and the threshold dose of epinephrine required to induce ventricular ectopic rhythms.
5. Atropine and tetraethylammonium offer no significant protection, although ergotamine does.
6. Sudden elevation of pressure in the denervated heart-lung preparation may cause ventricular premature contractions (bigeminal rhythm).
7. Epinephrine (in the absence of cyclopropane) prevents the bigeminal rhythm induced by pressure elevation in the heart-lung preparation.
8. Epinephrine plus a sufficient rise of pressure will induce multifocal ventricular discharges, but possibly not fibrillation, in the heart-lung preparation exposed to cyclopropane.

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# THE EVALUATION OF THE ANALGESIC ACTION OF METHADON ISOMERS AND OTHER ANALGESICS BY A NEW RAPID SCREENING METHOD

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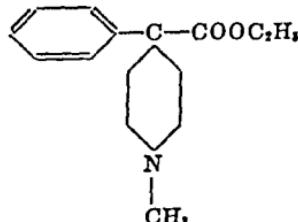
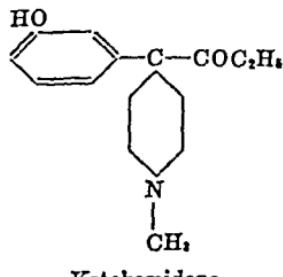
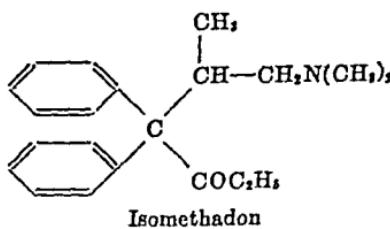
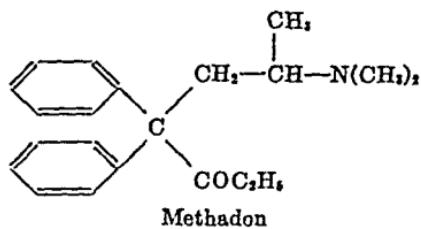
*Malbie Chemical Co., Morristown, N. J.*

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For the screening and comparative assay of analgesics, the heat stimulus test of Hardy & Wolff (1) as adapted to various animals such as the rat (2, 3, 4), dog (5), and guinea pig (6) has given reliable results. All methods now used require large groups of animals because of the variability of individual responses. They also necessitate laborious experimental effort and calculations so that the development of a rapid and statistically consistent method would be useful. The method described below has been devised for this purpose. It has been tried with well-known analgesics as well as with new drugs.

**MATERIAL AND METHODS.** *Material.* Two groups of male albino rats of the same strain were used, one group containing small rats (weighing 150 to 210 grams), and the other comprised of large rats (weighing 210 to 310 grams). Rats were maintained throughout the experiment on a diet of Purina Laboratory Chow.

Morphine sulphate and codeine sulphate (U.S.P. powder), merperidine hydrochloride, dl-Methadon (B) (amidone hydrochloride J. T. Baker—M.P.: 233.4–234°C. corr.),<sup>1</sup> dl-Methadon (W) (Winthrop—M.P: 230.8–232.9°C. corr.), l-Methadon, d-Methadon, l-Isomethadon (442C47), ketobemidone (Win. I.G. 10720),<sup>2</sup> and aminopyrine were used in water solutions in concentrations varying from 1:100 to 1:1000. Of interest is the relationship between the following structural formulas:



<sup>1</sup> dl-Methadon (B) was supplied by J. T. Baker Co., Philipsburg, New Jersey.

<sup>2</sup> dl-Methadon (W), l-Methadon, d-Methadon, l-Isomethadon, Ketobemidone have been available through the courtesy of Winthrop Co.

Acetylsalicylic acid was used either in suspension in water with 2 per cent acacia, or dissolved in water by the addition of NaHCO<sub>3</sub>, until a pH of 6.7 to 6.8 was reached.

**Apparatus.** The radiant heat apparatus of Ercoli and Lewis was used with the following additions: a thermocouple<sup>3</sup> to check that the temperature is constant, an insulated box enclosing the unit to maintain constant temperature, a mirror for a clearer view of the rat's back, an automatic device to start the timer with the opening of the shutter, and a bell alarm to signal completion of exposure time. Figure 1 is a diagrammatic drawing of the apparatus (sectional elevation).

**Procedure.** An area on the rat's back was shaved and the skin darkened with lamp black in preparation for exposure to the heat stimulus. In more recent experiments, however, a dull black brushing lacquer<sup>4</sup> was substituted successfully; this is clean, dries rapidly and does not cause irritation. The temperature of the radiant heat applied to the animal's skin

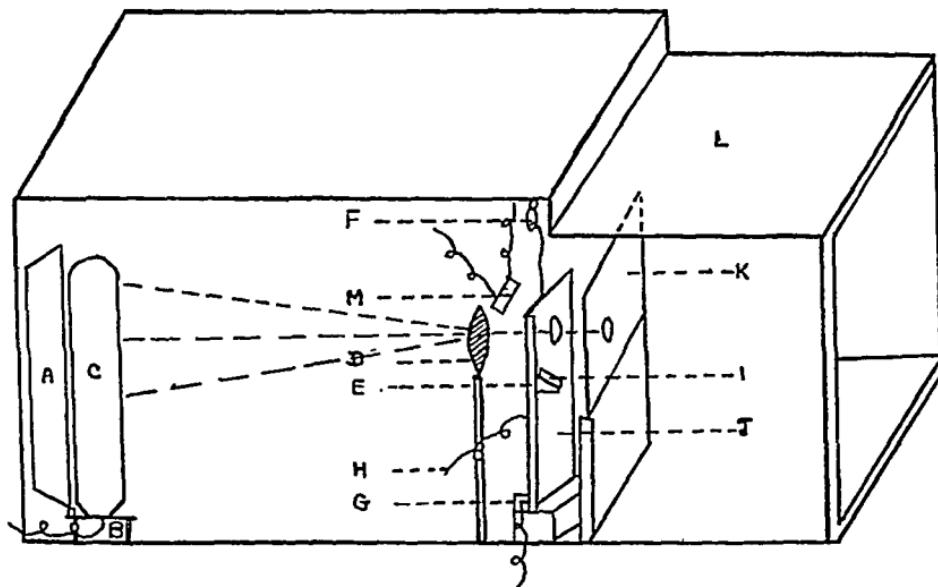


FIG. 1. DIAGRAMMATIC DRAWING OF RADIANT HEAT APPARATUS

(Sectional elevation with side panel removed.) A—Silver reflector; B—Connection to variac & voltmeter; C—1000 watt bulb; D—Lens; E—Shutter; F—Pulley for shutter; G—Contact which starts timer automatically when shutter is in open position; H—Connections to timer; I—Mirror to view muscle twitch of rat's back; J—Support for lucite; K—Lucite with aperture at focal point; L—Lucite top; M—Thermocouple.

was kept constant throughout the experiments as proved by a periodical checking. The intensity of heat stimulus was expressed in degrees centigrade (7) as read on the thermocouple instead of gm. cal./sec./cm.<sup>2</sup> delivered to the irradiated area (1).

Following Ercoli's (2) suggestion, the muscle twitch was used as the criterion of pain reaction. In addition, for the study of the analgesic effects of the antipyretics the flight reaction was used as the end point (2). The proportion of animals which failed to respond to the same intensity and duration of the painful stimulus was determined before and after the administration of analgesics.

**RESULTS.** The results are described in two sections. Section I deals with preliminary experiments concerning the choice of the animals, the intensity of

<sup>3</sup>This instrument was kindly devised by Dr. Richard Barry.

<sup>4</sup>Personal communication with Dr. Carl Pfeiffer.

heat stimulus and the length of exposure time for the best conditions of experimentation. Section II describes the application of the method to various analgesics.

*I. Preliminary experiments.* One hundred and ninety-two small rats (weight 150–210 grams) were used. Without medication, the skin was exposed to a constant intensity of heat expressed as 69°C., and the reaction time of each animal was recorded. Individual responses varied from two to ten seconds and results showed a normal frequency distribution, the mean of which was between three and four seconds, whereas it was much higher (six to eight seconds) in the case of seventy-two large rats (weight 210–310 grams). Therefore, only small rats were selected for our procedure.

Comparison of the reaction to three different intensities of heat, 62°C., 66°C., and 69°C., was made on a group of small rats before medication. Figure 2 shows

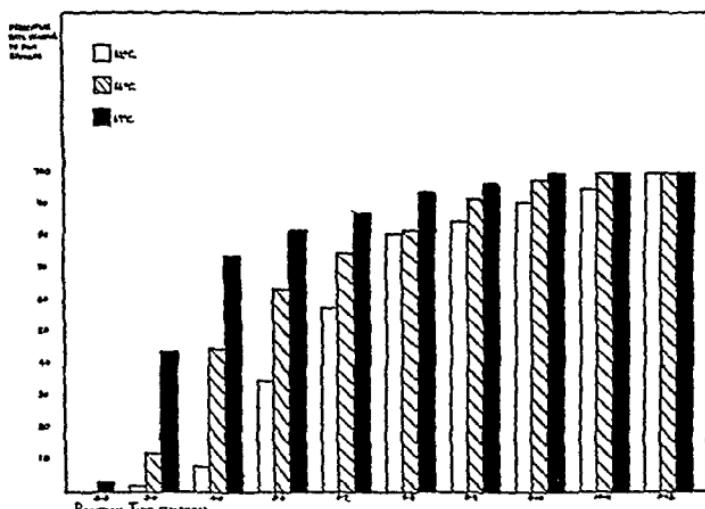


FIG. 2. FREQUENCY DISTRIBUTION OF UNTREATED RATS TO DIFFERENT HEAT STIMULI

the relationship of exposure time to the percentage of rats which responded to these various heat stimuli. It was found that 94 per cent of the rats reacted to a heat stimulus of 69°C. at an eight seconds exposure or less, 81 per cent reacted at 62°C. at eight seconds, and 35 per cent reacted at 62°C. with six seconds exposure.

In order to evaluate the statistical accuracy of the procedure using 69°C. and eight seconds exposure, three well-known analgesics (morphine, codeine and merperidine) were tested. Data were compared with results obtained using 62°C. with six seconds exposure and 62°C. with eight seconds exposure.

The standard error of E.D. 50 was estimated graphically according to Miller and Tainter (8). The goodness of fit was evaluated by the  $\chi^2$  test according to the recent graphic simplified method of Wilcoxon and Litchfield (9).<sup>5</sup> The

<sup>5</sup> We are indebted to Dr. J. T. Litchfield for his unpublished paper. Copies are available from American Cyanamid Co., Stamford, Conn.

inherent precision as described by Bliss and Cattell (10) was computed in terms of: 1—standard deviation of the logarithms of the individual effective doses ( $\lambda$ ) (Gaddum) (11), 2—slope of the curve ( $b = 1/\lambda$ ) (11, 12), 3—probability factor ( $P$ ) (13). Results are summarized in table I. The method using six seconds exposure at 62°C. is the most sensitive as proved by a smaller E.D. 50. It is, however, the least accurate. The inherent precision (10) favors somewhat the stimulus of eight seconds at 62°C. The eight seconds exposure at a temperature of 69°C., however, is more convenient and rapid inasmuch as it makes possible the use of most of the animals submitted to experimentation without the need of rejecting many of the less sensitive ones. For this reason it has been selected for rapid screening. If there are indications of low analgesic activity in the drugs tested, further investigation may be made with 62°C. at six seconds exposure.

*II. Application of the method to various analgesics.* For testing of analgesic activity the effect of any unknown was compared to morphine as a standard. Previous to the administration of analgesics all animals were tested at eight seconds exposure time and those which did not respond were rejected. Following

TABLE I

Degree of accuracy under different conditions of heat stimulus and exposure time

DRUG	NO. RATS	HEAT STIMULUS °C.	EXPOSURE TIME sec.	P	E.D. 50 mgs./kgm.	$\lambda$	SLOPE ( $b = 1/\lambda$ )
Morphine.....	96	69	8	.85	3.01 ± .21	.33	3.0
		62	6	.35	1.4 ± .24	.37	2.7
		62	8	.90	2.6 ± .50	.29	3.4
Codeine.....	144	69	8	.80	26 ± 10.5	.61	1.6
		62	6	.40	8.8 ± 3.3	.59	1.7
		62	8	.40	16 ± 4.1	.46	2.1
Merperidine....	72	69	8	1	21 ± 2.8	.30	3.3
		62	6	1	13 ± 2.7	.37	2.7
		62	8	1	23 ± 2.7	.30	3.3

the administration of the drug, the rats were submitted to the same stimulus and the proportion of animals which were protected was recorded. For each drug three or more doses in logarithmic progression were used on at least sixteen rats for each dose. Both the unknown drug and the standard could be tested in less than one hour.

1) Well-known analgesics. The method was applied to the well-known analgesics, codeine and merperidine, and compared to morphine. For establishment of the regression line, data were plotted on a Miller-Tainter graph paper (8). Figure 3 shows a linear relationship between the logarithm of the dose and the probit (13) of the rats which failed to react to the stimulus. The data on the observed precision are shown in table II.

Of interest is the fact that further testing of morphine and merperidine at the

"expected" E.D. 50 level gave the following results respectively: E.D. 53 and E.D. 47.

The duration of analgesia was estimated by determining the reaction to the test every half hour. Most drugs given at the E.D. 50 level showed a peak of action at thirty minutes (figure 4). The duration of analgesia, however, varied with the dose.

2) Methadon isomers and ketobemidone. Because of the scanty and contradictory data available concerning methadon isomers and ketobemidone, the analgesic potency of these drugs was tested by the above method. Figure 3 shows the dose response curves of these drugs. Table II compares their average effective doses with well-known analgesics. Figure 4 summarizes the ratio to morphine of these analgesics and the duration of their action.

TABLE II

*Statistical evaluation of the method as applied to methadon isomers and other analgesics*

DRUG	% OF RATS	P	AVERAGE EFFECTIVE DOSE (E.D. 50)	$\lambda$	SLOPE ( $b = 1/\lambda$ )
Morphine	352	.75	3.01 $\pm$ 0.21	.33	3.0
Codeine	144	.45	24 $\pm$ 4.7	.53	1.9
Merperidine	96	1.0	21 $\pm$ 2.8	.30	3.3
dl-Methadon (B)	96	1.0	2.5 $\pm$ 0.28	.31	3.2
dl-Methadon (W)	48	1.0	2.3 $\pm$ 0.35	.31	3.2
d-Methadon	48	.70	82 $\pm$ 0.80	.38	2.7
l-Methadon	48	.40	1 $\pm$ 0.32	.32	3.1
l-Isomethadon	104	1.0	3.6 $\pm$ 0.53	.44	2.2
Ketobemidone (Win IG-10720)	123	.07	2.9 $\pm$ 0.37	.32	3.1

An illustration of the consistency of the method was the testing of two samples of dl-Methadon received from different sources. The E.D. 50 of dl-Methadon (W) was 2.3 mgm./kgm. and that of dl-Methadon (B) 2.5 mgm./kgm. Moreover, dose-effect curves were quite parallel (figure 3).

3) Control. Tests with saline were made at random without the operator's knowledge. Analgesic effect was found in one out of every 60 rats only.

4) Comparison with results of other methods. Table III shows the ratio to morphine of the analgesics studied by this procedure as compared with data available in the literature both in various animal species and human beings. Our results agree with clinical data.

5) "Analgesic" activity of some antipyretic drugs. Data available in the literature concerning the "analgesic" effect of acetylsalicylic acid in laboratory animals are contradictory and sometimes unconvincing. The present method was applied to acetylsalicylic acid using different routes of administration and various vehicles. Results are summarized in table IV. Orally, both in suspension with 2 per cent acacia and in solution with NaHCO<sub>3</sub>, at the

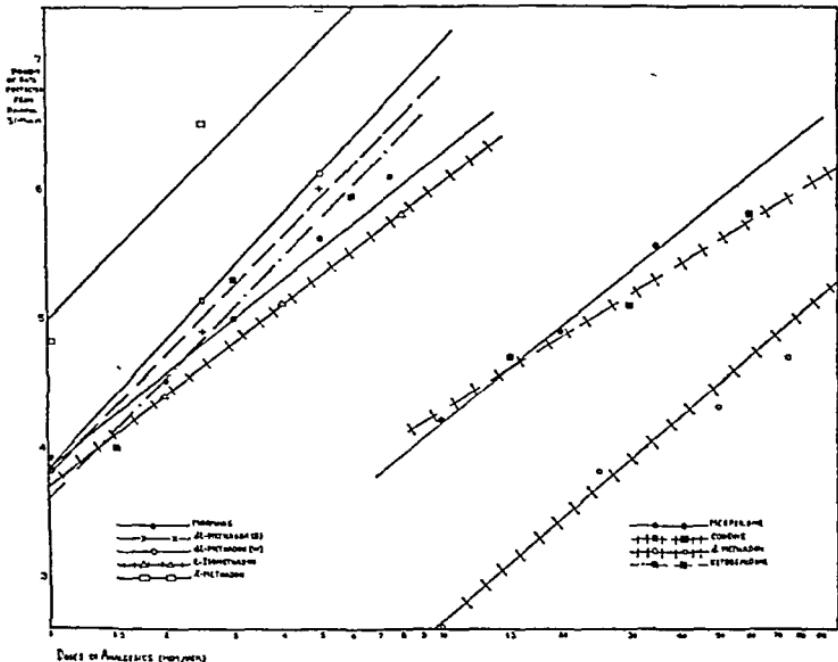


FIG. 3. DOSE-EFFECT CURVE OF METHADON ISOMERS AND OTHER ANALGESICS

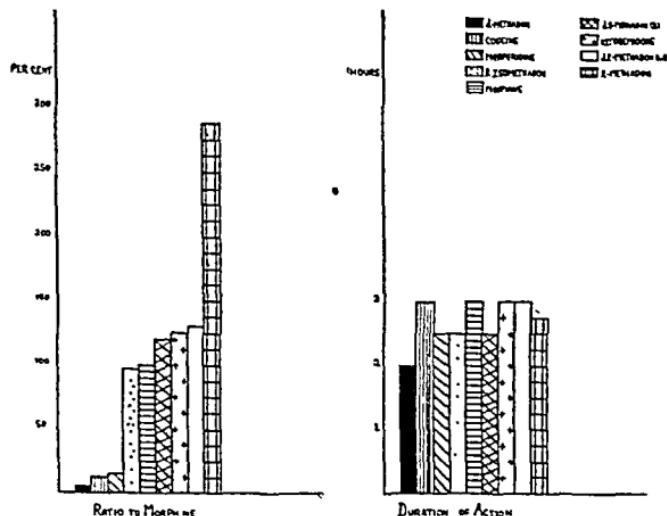


FIG. 4. RATIO TO MORPHINE AND DURATION OF ACTION OF METHADON ISOMERS AND OTHER ANALGESICS

dosage of 500 mgm. and 1000 mgm./kgm., acetylsalicylic acid did not decrease the reaction to pain.

Inasmuch as these negative results might be due to the route of administration, the drug was given intraperitoneally in solution with NaHCO<sub>3</sub>. No analgesic effect was observed even at the toxic dose level of 500 mgm./kgm.

When the more sensitive test (62°C. and six seconds exposure time) was used, however, it was found that there was some activity by the oral route and a slight but significant effect (31 per cent) by the intraperitoneal route. Controls with saline showed no effect.

Aminopyrine was not active until the toxic dose was reached when the muscle twitch was used as the end point. When the flight reaction was used as the end point a clear-cut effect was observed which, however, was not proportional to the dose (see table V). This confirms the results of Ercoli et al. (2).

**DISCUSSION.** The apparatus used to project radiant heat is essentially the same as that described by Ercoli (2) and has been found very convenient. In

TABLE III  
*Ratio of activity of analgesics to morphine by various screening methods applied to different animal species*

DRUG	RAT (TMIS METH- OD)	RAT	MOUSE	CAT	DOG	GUINEA PIG	MAN
Morphine	100	100	100	100	100	100	100
Codeine	12.5	14 (2)					10 (1)
Merperidine	13.5	40 (19) 16 (2, 21)	16 (21)			20-25 (6)	13.6 (17)
dl-Methadon (B)	120	200 (14)	200 (15)		200 (14)		150 (16)
dl-Methadon (W)	130	130 (24, 25)					200 (23)
d-Methadon	3	<10 (24, 25)					
l-Methadon	286	220 (24, 25)					300 (16)
l-Isomethadon	98	100 (24)					150 (16)
Ketobemidone	104	200 (14)	.				

order to eliminate the effects of air currents on the heat intensity, the whole apparatus was totally enclosed in an asbestos insulated box. Under these conditions, following a fifty-minute induction period, a constant temperature can be maintained at the exposure spot throughout the experiment. Following Slaughter and Wright's suggestions (7), the intensity of radiant heat was measured in °C. by the use of a thermocouple. By means of a mirror installed in the box, the back of the rat could be viewed by reflected light, thereby reducing the glaring rays of direct light which blur the operator's eyes in spite of the use of sunglasses. This device permits an unequivocal determination of the muscle twitch. In addition, the synchronization of the shutter and timer increased the accuracy of the experiment.

Previous methods using a heat stimulus have depended either upon the determination of the intensity of heat applied at a constant time of exposure (1, 5, 6) or upon the determination of the reaction time with a constant intensity of heat (2, 3, 4).

Such methods, however, are liable to great inaccuracy because of the variability of the responses of individual rats and necessitate the use of a larger group of animals. The present technique takes advantage of the inter-individual variation in sensitivity by taking into account the percentage of animals which react

TABLE IV  
"Analgesic" activity of acetylsalicylic acid

DOSE	VEHICLE	HEAT STIMULUS		NO. RATS	NO. PROTECTED	MORTALITY	HEAT STIMULUS		NO. RATS	NO. PROTECTED	MORTALITY
I. Oral route											
mgm./kgm.		°C.	sec.				°C.	sec.			
250	Acacia suspension	69	8				62	6	16	1/16	0
500				8	0	0			16	2/16	0
1000				8	0	0			16	1/16	1/16
250	Sol. + NaHCO <sub>3</sub>	69	8	8	0	0	62	6	16	2/16	2/16
500				8	0	0			22	1/22	1/22
1000				8	0	0			23	4/23	2/23
0	Saline								24	2/24	0
II. Intraperitoneal route											
125	Sol. + NaHCO <sub>3</sub>	69	8	8	0	0	62	6	32	5/32	0
250				16	1/16	0			32	6/32	0
500				24	1/24	1/24			32	9/32	1/32
750				16	1/16	1/16					
0	Saline								24	1/24	0/24

TABLE V  
"Analgesic" activity of aminopyrine  
Oral administration

DOSE	HEAT STIMULUS		NO. OF RATS SURVIVING	NO. PROTECTED MUSCULAR TWITCHING	NO. PROTECTED FLIGHT REACTION	DURATION OF ACTION	MORTALITY
mgm./kgm.	°C.	sec.				hrs.	
150	69	8	16	1/16	7/16	>2½	0
200	69	8	16	1/16	4/16	>2½	0
250	69	8	14	1/14	10/14	>2½	2/16
500	69	8	22	7/22	12/22	5½	7/29
750	69	8	12	4/12	6/12	5½	14/26

to a constant stimulus at different analgesic dose levels. The experimental data show that there is a linear relationship between the logarithm of the dose of the analgesic and the percentage of animals showing analgesic effect. An exception to this is aminopyrine.

Inasmuch as these negative results might be due to the route of administration, the drug was given intraperitoneally in solution with NaHCO<sub>3</sub>. No analgesic effect was observed even at the toxic dose level of 500 mgm./kgm.

When the more sensitive test (62°C. and six seconds exposure time) was used, however, it was found that there was some activity by the oral route and a slight but significant effect (31 per cent) by the intraperitoneal route. Controls with saline showed no effect.

Aminopyrine was not active until the toxic dose was reached when the muscle twitch was used as the end point. When the flight reaction was used as the end point a clear-cut effect was observed which, however, was not proportional to the dose (see table V). This confirms the results of Ercoli et al. (2).

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Merperidine	13.5	40 (19) 16 (2, 21)	16 (21)			20-25 (6)	13.6 (17)
dL-Methadon (B)	120	200 (14)	200 (15)		200 (14)		150 (16)
dL-Methadon (W)	130	130 (24, 25)					200 (23)
d-Methadon	3	<10 (24, 25)					
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L-Isomethadon	98	100 (24)					150 (16)
Ketobemidone	104	200 (14)	.				

order to eliminate the effects of air currents on the heat intensity, the whole apparatus was totally enclosed in an asbestos insulated box. Under these conditions, following a fifty-minute induction period, a constant temperature can be maintained at the exposure spot throughout the experiment. Following Slaughter and Wright's suggestions (7), the intensity of radiant heat was measured in °C. by the use of a thermocouple. By means of a mirror installed in the box, the back of the rat could be viewed by reflected light, thereby reducing the glaring rays of direct light which blur the operator's eyes in spite of the use of sunglasses. This device permits an unequivocal determination of the muscle twitch. In addition, the synchronization of the shutter and timer increased the accuracy of the experiment.

Previous methods using a heat stimulus have depended either upon the determination of the intensity of heat applied at a constant time of exposure (1, 5, 6) or upon the determination of the reaction time with a constant intensity of heat (2, 3, 4).

Such methods, however, are liable to great inaccuracy because of the variability of the responses of individual rats and necessitate the use of a larger group of animals. The present technique takes advantage of the inter-individual variation in sensitivity by taking into account the percentage of animals which react

TABLE IV  
"Analgesic" activity of acetylsalicylic acid

DOSE $\text{mg./kg.}$	VEHICLE	HEAT STIMULUS		NO. RATS	NO. PRO- TECTED	MORTAL- ITY	HEAT STIMULUS		NO. RATS	NO. PRO- TECTED	MORTAL- ITY
I. Oral route											
250	Acacia suspension	69	sec.				62	sec.	16	1/16	0
500				8	0	0			16	2/16	0
1000				8	0	0			16	1/16	1/16
250	Sol. + NaHCO <sub>3</sub>	69	sec.	8	0	0	62	sec.	16	2/16	2/16
500				8	0	0			22	1/22	1/22
1000				8	0	0			23	4/23	2/23
0	Saline								24	2/24	0
II. Intraperitoneal route											
125	Sol. + NaHCO <sub>3</sub>	69	sec.	8	0	0	62	sec.	32	5/32	0
250				16	1/16	0			32	6/32	0
500				24	1/24	1/24			32	9/32	1/32
750				16	1/16	1/16					
0	Saline								24	1/24	0/24

TABLE V  
"Analgesic" activity of aminopyrine  
Oral administration

DOSE $\text{mg./kg.}$	HEAT STIMULUS		NO. OF RATS SURVIVING	NO. PROTECTED MUSCULAR TWITCHING	NO. PROTECTED FLIGHT REACTION	DURATION OF ACTION	MORTALITY
150	69	sec.	16	1/16	7/16	>2½ hr.	0
200	69	sec.	16	1/16	4/16	>2½ hr.	0
250	69	sec.	14	1/14	10/14	>2½ hr.	2/16
500	69	sec.	22	7/22	12/22	5½ hr.	7/29
750	69	sec.	12	4/12	6/12	5½ hr.	14/26

to a constant stimulus at different analgesic dose levels. The experimental data show that there is a linear relationship between the logarithm of the dose of the analgesic and the percentage of animals showing analgesic effect. An exception to this is aminopyrine.

The method described is rapid, uses a minimum of time-consuming calculations and requires no special skill in its performance. Besides the inherent precision of the method, its reliability may be proved by the fact that the relative potencies of various analgesics agree with data of other authors (2, 5, 14, 15) and moreover of clinical experiments (1, 16, 17, 23). An exception, however, is acetylsalicylic acid, the "analgesic" effect of which cannot be detected by our present method. The testing of this drug in laboratory animals poses a challenge. Data published are conflicting. Winder (6) detected pain threshold raising properties in the guinea pig; Andrews and Workman (5) in the dog; Hart (4) and D'Amour (4) in the rat; Bonnycastle in the rat after potentiation by codeine (18); and Davies (19) following intravenous route only. On the contrary, Ercoli (2) and Goetzel (20) in the rat and Woolfe (21) and Kueter and Richards (22) in the mouse, were unable to observe any analgesic effect.

Reasons for such disagreements and for the contrast between results of laboratory and clinical tests have been discussed by many authors (6, 20, 21, 22). Another explanation is that acetylsalicylic is not active enough to alleviate an intensity of pain as great as the one used in our procedure. Proceeding on this assumption, a less intense pain stimulus (62°C., six seconds exposure) was employed and under these circumstances a small but clear-cut effect was observed particularly after intraperitoneal administration. This would agree with the therapeutic limitations of aspirin to low grade pain.

The increased sensitivity of this procedure which uses a less intense heat stimulus has been confirmed by other experiments (table I). However, because of its lower accuracy and the necessity of rejecting many of the less sensitive animals its use seems limited to the evaluation of minor analgesics.

#### SUMMARY

1. A simple and accurate method is described by which analgesics may be rapidly screened. It has been applied to morphine, codeine, merperidine, methadon isomers, ketobemidone, aminopyrine, and acetylsalicylic acid.

2. This method is based on an all-or-none response towards a pain heat stimulus of constant intensity and duration. A linear relationship exists between the logarithm of the doses of analgesic and the probit of rats failing to respond to the pain stimulus.

3. Statistical analysis of the data shows that the method has satisfactory inherent precision and yields reproducible results which are in agreement with clinical experience.

4. Evaluated by this method the two opiates tested and merperidine have the following average effective dose after subcutaneous administration expressed in mgm./kgm. of rat: morphine, 3.01; codeine, 24; merperidine, 21. The peak of action is thirty minutes. Using the average effective dose, the duration of analgesic activity of merperidine is two and one-half hours, and of morphine and codeine, three and one-half hours.

5. Methadon, its isomers, and ketobemidone show the following E.D. 50: dl-Methadon (B) 2.5 mgm./kgm., dl-Methadon (W) 2.3 mgm./kgm., d-Methadon

82 mgm./kgm., l-Methadon 1.05 mgm./kgm., l-Isomethadon 3.5 mgm./kgm., and ketobemidone 2.9 mgm./kgm. The duration of analgesic activity is more than two and one-half hours except for d-Methadon where it is less than two hours.

6. Among minor analgesics, aminopyrine is active only when the flight reaction is used as the end point. No linear dose-effect relationship can be observed. Acetylsalicylic acid given orally or intraperitoneally is inactive by this method. If a smaller intensity of heat is used as painful stimulus, however, a slight but definite "analgesia" is observed.

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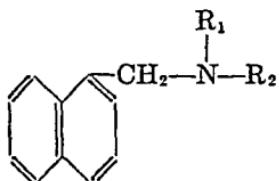
# ADRENERGIC BLOCKING DRUGS: II. ANTAGONISM OF HISTAMINE AND EPINEPHRINE WITH N-(2-HALOALKYL)-1-NAPHTHALENE-METHYLAMINE DERIVATIVES

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In previous reports (1-6) we have indicated that antagonism of histamine and epinephrine was effected by certain N-(2-haloalkyl)-1-naphthalenemethylamine derivatives. An extensive series of derivatives which were synthesized and supplied to us by Drs. G. Rieveschl, Jr., R. W. Fleming and W. R. Coleman<sup>2</sup> have been tested by several means in order to determine the quality of pharmacological actions exerted by structurally related compounds. The following type formula indicates the chemical structure of the compounds studied:



R<sub>1</sub> = alkyl, alkyloxy or  
chloro-alkyl  
R<sub>2</sub> = bromo- or chloro-  
alkyl

Chemically, these compounds are related to Dibenamine (N-(2-chloroethyl)dibenzylamine·HCl), a compound which exerts adrenergic blocking action but which is almost devoid of antihistamine action (7, 8). The new compounds investigated were first tested in mice to determine their ability to diminish the toxicity of epinephrine which constituted presumptive evidence of adrenergic blocking activity (1, 6). A majority of the compounds were tested by a second screening procedure which permitted selection of those compounds which exerted antihistamine action as revealed by the ability to diminish bronchospasm in guinea pigs subjected to an histamine aerosol; several compounds were also tested for anti-anaphylactic activity. Further evidence of adrenergic blocking action and antihistamine action was secured by demonstrating epinephrine reversal and diminution of the depressor action of histamine, respectively, in anesthetized, intact dogs. Evidence of an atropine-like action was based solely on a comparison of the depressor responses to acetylcholine before and after injection of the compounds in dogs.

A. *Reduction of epinephrine toxicity in mice.* We recently presented evidence (6) that the majority of known adrenergic blocking agents, when administered orally to mice, diminished the toxicity of injected epinephrine. Furthermore, numerous other types of drugs failed to reduce the toxicity of epinephrine in

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mice. Thus, the demonstration that a compound reduces epinephrine toxicity in mice constitutes a presumptive test for adrenergic blocking activity.

TABLE I  
*The antagonism of epinephrine and histamine by N-(*o*-haloalkyl)-1-naphthalenemethylamine derivatives*

Comp. No.	R <sub>1</sub>	R <sub>2</sub>	REDUCTION OF EPINEPHRINE TOXICITY IN MICE*, ORAL DOSE $\pm$ S.E.		ACUTE TOXICITY ORAL, MICE LD <sub>50</sub> $\pm$ S.E.	ORAL LD <sub>50</sub> / EFFECTIVE ORAL DOSE AGAINST EPINEPHRINE	DIMINUTION OF HISTAMINE-INDUCED BRONCHIO-SPASMS IN GUINEA PIGS M.E.D., S.U.C.
			mgm./kgm.	mgm./kgm.			
1	Methyl	2-chloroethyl	17.7 $\pm$ 2.5	1118 $\pm$ 85	63	0.05	
2 (SY-14)	Ethyl	2-chloroethyl	5.7 $\pm$ 0.6	725 $\pm$ 57	127	0.025	
3 (SY-28)	Ethyl	2-bromoethyl†	5.6 $\pm$ 0.7	457 $\pm$ 42	82	0.03	
4	n-Propyl	2-chloroethyl	3.8 $\pm$ 0.5	1080 $\pm$ 123	284	1.0	
5	Isopropyl	2-chloroethyl	3.0 $\pm$ 0.3	1104 $\pm$ 77	368	<3.0	
6	Allyl	2-chloroethyl	6.7 $\pm$ 0.9	1279 $\pm$ 89	191	3.0	
7 (SY-10)	n-Butyl	2-chloroethyl	4.5 $\pm$ 0.6	1070 $\pm$ 94	237	12.5	
8	Sec.-butyl	2-chloroethyl	6.8 $\pm$ 0.7	1202 $\pm$ 61	178	12.5	
9	Isobutyl	2-chloroethyl	16.8 $\pm$ 1.9			>12.5	
10	n-Amyl	2-chloroethyl	14.0 $\pm$ 2.4	1413 $\pm$ 49	101	>12.5	
11 (SY-63)	n-Hexyl	2-chloroethyl	8.8 $\pm$ 1.0	>2000	>227	>25.0	
12	2-Methoxyethyl	2-chloroethyl	4.9 $\pm$ 0.8	800 $\pm$ 91	163	1.5	
13	2-Chloroethyl	2-chloroethyl	15.5 $\pm$ 3.0			25.0	
14	Ethyl	2-chloroethyl	36.0 $\pm$ 3.4				
15	Ethyl	2-hydroxyethyl	>50.0			>25.0	
16	N-(2-chloroethyl)-N-ethyl-2-naphthalenemethylamine		>25.0				
17	N-(2-chloroethyl)-N-ethyl-5,6,7,8-tetrahydro-2-naphthalenemethylamine		>25.0				
18	4-chloro-N-(2-chloroethyl)-N-ethyl-1-naphthalene-methylamine		25.0			>12.5	

\* The hydrobromide salt; all others as hydrochlorides.

† The effective oral dose represents the calculated amount required to reduce mortality of mice from 67.0 to 33.5 per cent following intraperitoneal injection of a standard dose of epinephrine (cf. ref. 6 for details).

The results obtained with this method of screening the compounds in the series of N-(*o*-haloalkyl)-1-naphthalenemethylamine derivatives are presented in table 1. Two or more doses of each compound were administered orally to groups of 20 mice two hours before intraperitoneal injection of an amount of epinephrine hydrochloride which killed 67.0 per cent of untreated mice. The stated calculated doses are those required to protect 50 per cent of mice which

otherwise would have died (for details see ref. 6). Examination of the effective doses reveals that the alkyl derivatives (nos. 1 to 12) were all effective in doses ranging from 3.0 to 18.0 mgm./kgrn. (0.06 to 0.36 mgm., orally, per 20 gram mouse). The low magnitude of the effective doses clearly indicates that many of the compounds possess high potency. The methyl compound (no. 1) proved less active than most of the alkyl homologues as indicated by the comparatively large dose required to antagonize epinephrine in mice and by the fact that a dose of 5.0 mgm./kgrn., intravenously, was required to reverse the action of epinephrine in a dog experiment. In general, the lower alkyl homologues (ethyl to sec.-butyl, and n-hexyl; nos. 2 to 8, and 11) were the most effective in mice. Further considerations of activity are discussed in the sections below which concern epinephrine reversal in dogs and structure-activity relationships.

B. *Acute toxicity.* The acute toxicity of numerous compounds in the series was determined following oral administration of two to four doses to groups of 20 mice (19 to 21 grams; both sexes) which were observed for five days for symptoms and incidence of mortality. For oral administration, a 10 per cent aqueous suspension of each compound was made in a 2 per cent suspension of gum acacia. LD<sub>50</sub> values were estimated after plotting percentage mortality on log.dose-probit charts (9). Included in table 1 are the ratios of the oral LD<sub>50</sub> to the calculated oral doses which antagonized epinephrine toxicity, the doses in each instance being administered to non-fasted mice. The ratios indicate a wide range between the doses exerting epinephrine antagonism and the toxic doses. In most instances, the ratio is of such magnitude as not to eliminate an individual compound from further study since other chemical, pharmacological and toxicological properties could well be of great importance.

None of the compounds, in oral doses of 1.5 to 50.0 mgm./kgrn. used in attempts to reduce epinephrine toxicity, produced any symptoms in mice during the two hours before epinephrine was injected. The large doses of compounds used in the toxicity experiments were practically devoid of stimulant action on the somatic nervous system as indicated by the absence of hyperexcitability, tremors and convulsions. Some ataxia and depression was noted coincident with dyspnea in those mice which succumbed. The majority of deaths occurred within one to four hours after administration of the compounds; opening of the thorax immediately after respiratory failure revealed that the heart was still beating. Some mice remained depressed for 24 to 48 hours, and a few dead mice were found at these time intervals, but seldom later.

C. *Diminution of histamine-induced bronchospasm in guinea pigs.* Attention has been drawn to the fact that all of the known antihistamine drugs either enhance or diminish the pressor responses to epinephrine in dogs (3). Obviously, compounds known to exert antihistamine action should be tested for adrenergic blocking action and vice versa. Various compounds from the chemical series under investigation were injected subcutaneously in groups of twelve guinea pigs 30 minutes before subjecting them to an histamine aerosol which induced mortality in 92 per cent of an equivalent control group. The doses included in table 1 are the approximate minimal effective doses required to protect a significant number of animals from death referable to bronchoconstriction.

The data reveal that the lower alkyl homologues, i.e., the methyl (no. 1) and specially the ethyl (nos. 2 and 3), were markedly effective in antagonizing histamine. The latter compounds proved to be about as effective as pyranisamine (Neo-antergan) (4) when a direct comparison was made. Potency, as measured by diminution of histamine-induced bronchospasm, decreased rapidly as additional carbon atoms were added to the alkyl chain (nos. 4 to 11). It is at once apparent that the ability to antagonize the effect of histamine on bronchioles of guinea pigs is not proportional to the ability to decrease epinephrine toxicity in mice.

TABLE 2  
Control of anaphylaxis in guinea pigs

COMPOUND	DOSE S.C. mgm./ kgm.	MORTALITY			SEVERITY OF SYMPTOMS IN SURVIVORS
		Ratio	%	P*	
Untreated controls		10/20	50		4—mild 6—moderate
Benadryl·HCl	3.0	0/7	0	<0.01	7—mild
	0.5	1/9	11	0.05	6—mild; 2— moderate
Compound 2. (SY-14). N-(2-chloroethyl)-N-ethyl-1-naphthalenemethylamine·HCl	0.15	0/7	0	<0.01	7—all mild
Compound 3. (SY-28). N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine·HBr	0.20	0/7	0	<0.01	7—all mild
	0.03	0/7	0	<0.01	5—mild; 1—severe
Compound 5. N-(2-chloroethyl)-N-isopropyl-1-naphthalenemethylamine·HCl	1.5	0/10	0	<0.01	1—moderate 9—mild; 1— moderate

\* P values from Fisher's Table; value of 0.05 or less indicates a significant difference.

D. *Alleviation of anaphylactic symptoms in guinea pigs.* Those compounds which diminish bronchospasm due to injected histamine should prove effective in decreasing bronchospasm occurring in anaphylaxis which is referable to histamine released from body tissues. Three compounds from the series which exhibited a marked ability to protect guinea pigs in the histamine aerosol experiments were tested to determine their protective action in anaphylaxis (table 2).

Each guinea pig was sensitized by an intraperitoneal injection of 0.5 cc. of normal horse serum. Eighteen days later, test compounds were administered subcutaneously to all but control animals 30 minutes before the injection of 1.0 cc. of the antigen intracardially. The antihistamine drug, diphenhydramine·HCl (Benadryl), was employed as a reference drug and proved effective under the experimental conditions in decreasing mortality due to anaphylaxis (table 2). N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine·HBr (no. 3) and the chloroanalogue (no. 2) were both effective in reducing mortality and preventing all but mild symptoms when used in doses of 0.20 and 0.15 mgm./kgm., respectively,

which are approximately molar equivalents. The former compound reduced mortality when a dose of only 0.03 mgm./kgm. was employed, thus demonstrating high potency with respect to protection against anaphylaxis and protection against histamine aerosol (table 1). Protection against anaphylaxis was also demonstrated with the isopropyl homologue (no. 5) although smaller doses were not used to determine the degree of activity.

E. *Epinephrine reversal and histamine antagonism in dogs.* Most of the compounds which were found to reduce epinephrine toxicity in mice were injected intravenously in dogs anesthetized with pentobarbital sodium in an attempt to demonstrate epinephrine reversal and thus secure support for the belief that the compounds acted as adrenergic blocking agents. A few compounds which were non-effective or of low effectiveness in antagonizing epinephrine in mice were also administered to dogs since the failure to demonstrate epinephrine reversal readily in dogs would support the reliability of the mouse screening method. These experiments in dogs were so designed also to yield information concerning histamine antagonism and atropine-like action.

In brief, the dog experiments were conducted as follows: Two intravenous injections of epinephrine hydrochloride (10 gamma), acetylcholine bromide (25 gamma) and histamine diphosphate (50 gamma) were made prior to administration of a test drug. The test drug was then injected slowly (2 minutes) intravenously. After waiting 10 minutes for distribution of the compound, onset of action, and stabilization of blood pressure, each of the pressor and depressor drugs was again administered twice and such injections were repeated beginning at 60 and again at 120 minutes after injection of the test compound. Comparison of the paired pressor and depressor responses occurring 10 to 30, 60 to 90, and 120 to 150 minutes after injection of the test compound with those occurring before constituted the means of demonstrating presence or absence of blocking or reversal of the pressor response to epinephrine and diminution of the depressor action of histamine and acetylcholine. The mid-portion of a record of such an experiment is presented in figure 1.

Preliminary experiments revealed that all of those compounds which antagonized epinephrine in mice (table 1) were effective in causing epinephrine reversal when injected intravenously in dogs. Furthermore, the several compounds which were inactive or exhibited low activity in mice did not reverse the pressor action of epinephrine, or did so only when doses of 10.0 mgm./kgm. were injected (nos. 15 to 19). Thus, the mouse screening method is of definite value in selecting and evaluating adrenergic blocking compounds.

In table 3 are presented quantitative data relating to the effects of several of the alkyl derivatives on vascular responses to epinephrine, histamine and acetylcholine. Further studies with the ethyl derivatives (nos. 2 and 3) were indicated because of the high potency of each with respect to epinephrine antagonism and histamine antagonism. The N-(2-chloroethyl)-N-ethyl derivative (no. 2 or SY-14), in a dose of 3.0 mgm./kgm., intravenously, consistently induced epinephrine reversal and definitely diminished the depressor responses to histamine for at least 150 minutes. The bromo-analogue (no. 3 or SY-28) exhibited

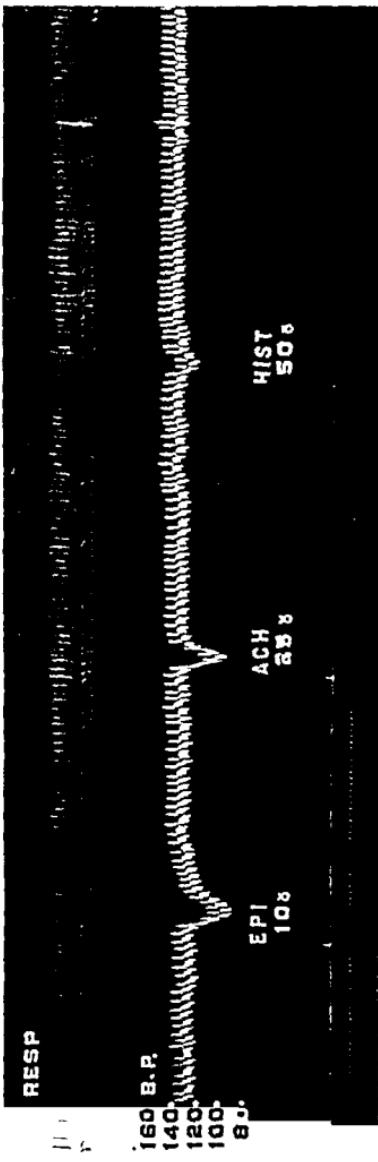
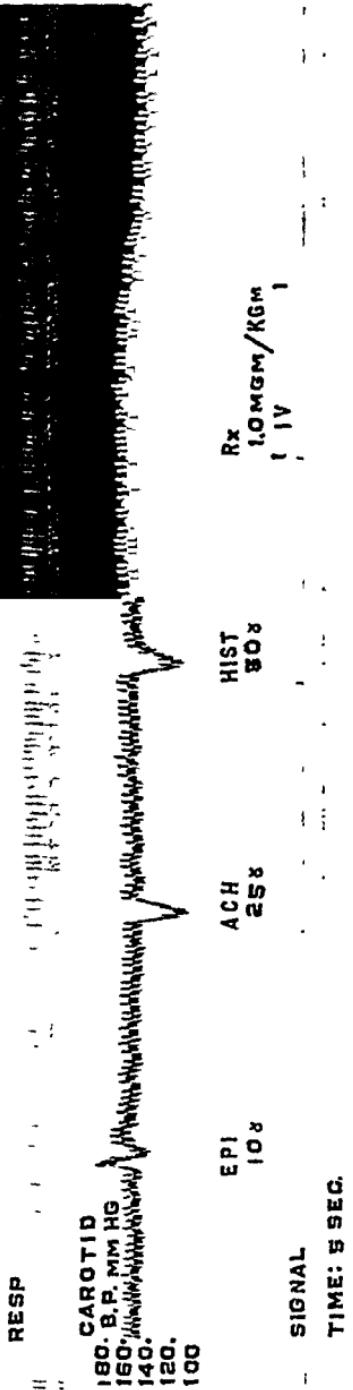


FIG. 1. EFFECT OF N-(2-monoethyl)-N-ethyl-1-naphthalenemethylamine HBr (SY-28) ON BLOOD PRESSURE RESPONSES IN THE DOG ANESTHETIZED WITH PENTOTHALNALETHAL. SODIUM

Upper record shows the second series of injections of epinephrine, acetylcholine and histamine before slow intravenous injection of the compound in a dose of 1.0 mgm./kgm. (at Rx). Note slow decrease in blood pressure.

Lower record was begun 10 minutes later; note reversal of the response to epinephrine, slight diminution of depressor response to acetylcholine and almost complete blocking of the depressor response to histamine. Similar effects were obtained 60 to 110 and 120 to 150 minutes after treatment.

TABLE 3  
Effect of several N-(*S*-haloalkyl)-1-naphthalenemethylamines on pressor responses to epinephrine and depressor responses to histamine and acetylcholine in dogs

COMP. NO.	Dose, I.V.	EPINEPHRINE HYDROCHLORIDE 10 GAMMA				HISTAMINE DIHClO <sub>4</sub> 50 GAMMA				ACETYLCHOLINE BROMIDE 25 GAMMA							
		After treatment mean difference $\pm$ S.E. $\dagger$		After treatment mean difference $\pm$ S.E.		Control mean Depressor response		Control mean Depressor response		Control mean Depressor response		Control mean Depressor response					
		No. Ext. Pressor Spikes	No. Ext. Depressor Spikes	10-30 min.	P	10-30 min.	P	120-150 min.	P	10-30 min.	P	120-150 min.	P				
2 (SY-11)	3.0 4	46.0 84.0	$\pm$ 11.5	<0.01 92.2	$\pm$ 13.9	<0.01		39.2 24.2	$\pm$ 4.6	0.01 21.3	$\pm$ 2.4	<0.01	33.2 11.0	$\pm$ 6.5	0.01 11.1	$\pm$ 6.5	0.05
3 (SY-28)	0.2 7 1.0 7 3.0 4	34.1 40.3 42.0 78.7 40.3 71.3	$\pm$ 6.3 $\pm$ 9.2 $\pm$ 4.9	<0.01 33.4 <0.01 73.0 0.02 67.2	$\pm$ 8.7 $\pm$ 10.1 $\pm$ 15.6	<0.01 <0.01 0.02		45.0 20.1 41.0 25.0 39.2 27.2	$\pm$ 2.9 $\pm$ 2.6 $\pm$ 7.3	<0.01 6.6 <0.01 21.6 0.04 30.0	$\pm$ 3.9 $\pm$ 3.2 $\pm$ 7.1	0.15 <0.01 0.03	37.4 11.1 41.0 11.2 39.2 17.7	$\pm$ 5.1 $\pm$ 3.7 $\pm$ 6.8	0.06 7.1 0.02 8.7 0.07 24.2	$\pm$ 5.0 $\pm$ 3.3 $\pm$ 6.8	0.02 0.2 0.01 0.01 0.01 0.01
7 (SY-10)	3.0 4	38.2 34.0	$\pm$ 9.9	0.05 32.7	$\pm$ 10.6	0.05		50.7 22.0	$\pm$ 4.5	0.02 20.5	$\pm$ 4.0	0.01	33.2 4.6	$\pm$ 3.7	0.03 8.5	$\pm$ 3.2	0.07
11 (SY-03)	3.0 5	34.2 33.0	$\pm$ 7.1	0.01 30.2	$\pm$ 8.0	0.02		42.8 13.0	$\pm$ 1.9	0.01 17.0	$\pm$ 4.1	0.01	51.8 13.1	$\pm$ 2.8	0.01 18.0	$\pm$ 7.3	0.06

\* Pressor and depressor responses and mean differences from control values are all expressed as mm. Hg.

† Mean differences greater than the control pressor responses indicate epinephrine reversal, and those which approximately equal control pressor responses indicate marked diminution or blocking. No pressor spikes greater than 5 mm. Hg preceded the depressor responses to epinephrine in dogs receiving 3.0 mgm./kgm. of compounds 2 and 3, although pressor spikes of 4 to 32 mm. Hg occurred in 2 of 7 dogs receiving 1.0 mgm./kgm. of compound 3. Pressor spikes ranging to 35 mm. Hg frequently preceded depressor responses to epinephrine in animals treated with compounds 7 and 11 (3.0 mgm./kgm.) and compound 3 (0.2 mgm./kgm.).

‡ Data related to responses at the 60 to 90 minute interval are omitted since they never varied significantly from those recorded for the 120 to 150 minute interval.

§ Complete blocking of depressor responses to histamine or acetylcholine never occurred since mean differences never equalled the control values.

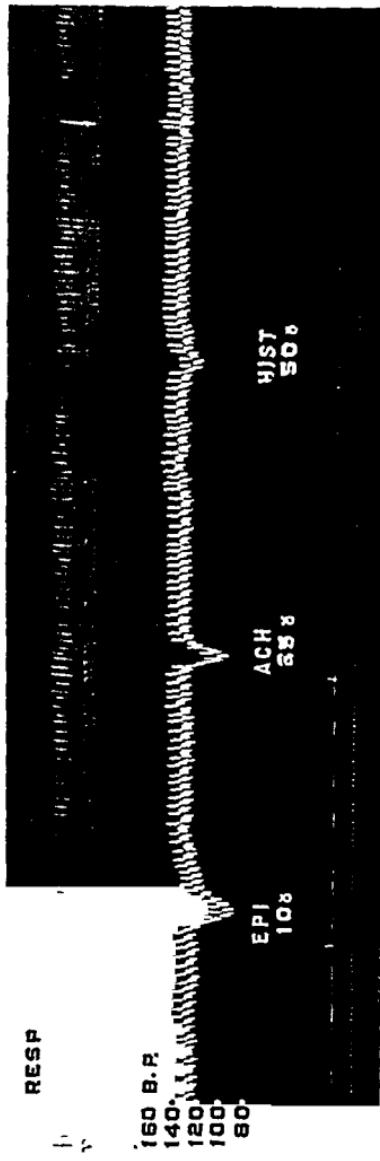
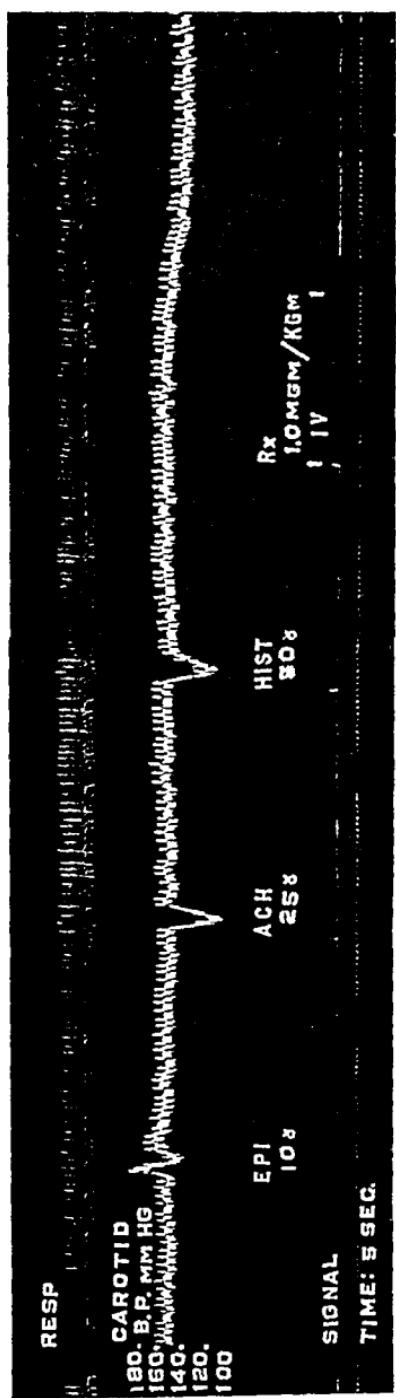


Fig. 1. Effect of N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine HBr (SY-28) on Blood Pressure Responses in the Dog ANESTHETIZED WITH PENTOBARBITAL SODIUM

Upper record shows the second series of injections of epinephrine, acetylcholine and histamine before slow intravenous injection of the compound in a dose of 1.0 mgm./kgm. (at Rx). Note slow decrease in blood pressure.

Lower record was begun 10 minutes later; note reversal of the response to epinephrine, slight diminution of depressor response to acetylcholine and almost complete blocking of the depressor response to histamine. Similar effects were obtained 90 and 120 to 150 minutes after treatment.

TABLE 3  
Effect of several *N*-(*2-haloalkyl*)-1-naphthalenemethylamines on pressor responses  
in dogs

cont. no.	Dose I.V.	EPINEPHRINE HYDROCHLORIDE 10 GAMMA				INOSITOL DIPHOSPHATE 50 GAMMA				ACRYLICOLINE BROMIDE 25 GAMMA					
		After treatment mean difference $\pm$ S.E. <sup>†</sup>				After treatment mean difference $\pm$ S.E. <sup>‡</sup>				After treatment mean difference $\pm$ S.E. <sup>§</sup>					
		Control Depressor response, mean difference $\pm$ S.E. <sup>  </sup>	10-30 min.	P	120-150 min.	Control Depressor response, mean difference $\pm$ S.E. <sup>  </sup>	10-30 min.	P	Control Depressor response, mean difference $\pm$ S.E. <sup>  </sup>	10-30 min.	P	Control Depressor response, mean difference $\pm$ S.E. <sup>  </sup>	P		
2 (SY-14)	3.0	40.0	34.0 $\pm$ 11.5	<0.01	92.2 $\pm$ 13.0	<0.01	39.2	24.2 $\pm$ 4.6	0.01	21.3 $\pm$ 2.4	<0.01	33.2	11.0 $\pm$ 6.5	0.2	
3 (SY-28)	0.2	7	34.1	40.3 $\pm$ 6.3	<0.01	33.4 $\pm$ 8.7	<0.01	45.0	20.1 $\pm$ 2.0	<0.01	6.0 $\pm$ 3.9	0.15	37.4	11.1 $\pm$ 5.1	0.06
	1.0	7	42.0	78.7 $\pm$ 9.2	<0.01	73.0 $\pm$ 10.1	<0.01	41.0	25.0 $\pm$ 2.6	<0.01	22.6 $\pm$ 3.2	<0.01	41.0	11.2 $\pm$ 3.7	0.02
	3.0	4	40.3	71.3 $\pm$ 4.9	0.02	67.2 $\pm$ 15.6	0.02	39.2	27.2 $\pm$ 7.3	0.01	30.0 $\pm$ 7.1	0.03	39.2	17.7 $\pm$ 6.8	0.07
7 (SY-10)	3.0	4	38.2	31.0 $\pm$ 9.0	0.05	32.7 $\pm$ 10.6	0.05	50.7	22.0 $\pm$ 4.5	0.02	20.5 $\pm$ 4.0	0.01	33.2	4.6 $\pm$ 3.7	0.03
11 (SY-63)	3.0	5	34.2	33.0 $\pm$ 7.1	0.01	30.2 $\pm$ 8.0	0.02	42.8	13.0 $\pm$ 1.9	0.01	17.0 $\pm$ 4.1	0.01	51.8	13.1 $\pm$ 2.8	0.01

mean differences from control values are all expressed as min. Hg.

• Pressor and depressor responses to central nervous system responses indicate encephalopressor reversal, and those which approximately equal control pressor

<sup>†</sup> Mean differences greater than the control pressor responses indicate marked diminution or blocking. No pressor spikes greater than 5 mm. Hg preceded the depressor responses to epinephrine in dogs receiving 3.0 mgm./kgm. of compounds 2 and 3, although pressor spikes of 4 to 32 mm. Hg occurred in 2 of 7 dogs receiving 1.0 mgm./kgm. of compound 3. Pressor spikes ranging to 35 mm. Hg frequently preceded depressor responses to epinephrine in animals treated with compounds 7 and 11 (3.0 mgm./kgm.) and compound 3 (0.2 mgm.).

<sup>t</sup> Data related to responses at the 60 to 80 minute interval are omitted since they never varied significantly from those occurring at the 40 to 60 minute interval.

similar activity at doses of 1.0 and 3.0 mgm./kgm. At the low dose of 0.2 mgm./kgm. it blocked and sometimes reversed effects of epinephrine during the first hour and only blocked thereafter, whereas it diminished depressor responses to histamine for only 10 to 30 minutes. This indicates that epinephrine antagonism was more prolonged than histamine antagonism. Since doses of 0.2 and 1.0 mgm./kgm. induced epinephrine reversal and the higher dose diminished or blocked several sympathetic adrenergic nervous effects (5, 10), it is apparent that the available information warrants the conclusion that the compound is indeed a potent adrenergic blocking agent, although no more potent than the chloro-derivative (no. 2) if compared on a molecular basis. Evidence of long duration of action was indicated by the fact that epinephrine antagonism was demonstrated in three unanesthetized dogs 18 hours after intravenous injection of the compound in a dose of 3.0 mgm./kgm.

Whereas the ethyl derivatives readily induced epinephrine reversal, the higher alkyl derivatives in the series of chloroethylamines (n-butyl or no. 7 and n-hexyl or no. 11) only blocked the pressor responses to epinephrine when the compounds were injected in doses of 3.0 mgm./kgm. (table 3). With each of the four compounds, evidence of blocking or reversal of epinephrine and of diminution of histamine effects was apparent ten minutes after treatment at which time the first tests were conducted. In addition to early onset of action, there was evidence of prolonged action, i.e., antagonisms persisted for the duration of the experiments (150 minutes). In dogs, potency with respect to epinephrine antagonism appeared greater with the ethyl derivatives (nos. 2 and 3) than with the n-butyl (no. 7) and n-hexyl (no. 11) derivatives; no clear indication of differences in potency was apparent with these compounds in the experiments with mice.

It is of interest to note that vascular responses to histamine in dogs appeared to be more susceptible to diminution than the bronchiolar responses to histamine in guinea pigs. Intravenous administration of comparatively small doses of the n-butyl and n-hexyl compounds consistently diminished depressor responses to histamine in dogs throughout the experimental period (table 3) whereas it required a subcutaneous dose of 12.5 mgm./kgm. of the n-butyl compound to reduce histamine-induced bronchospasm in guinea pigs (table 1) and the n-hexyl compound failed to exhibit activity at a dose of 25.0 mgm./kgm. It is possible that these compounds exhibit some selectivity of antihistamine action on one or more components of the vascular system.

Following intravenous injection of adrenergic blocking compounds, arterial blood pressure was usually reduced 15 to 35 per cent of the original within 10 to 60 minutes, with a tendency for partial recovery to control levels during the following 90 minutes. Decreases in blood pressure exceeding 10 per cent of the control level were not observed with comparable or even larger doses of the inactive compounds in the series (nos. 15 and 16). Tentatively, the decreased blood pressure is ascribed to diminution or blocking of vasoconstrictor tonus of adrenergic origin. Admittedly, the diminished level of arterial pressure may account, in part, for some of the diminution in vascular responses to epinephrine, histamine and acetylcholine.

*F. Evidence of atropine-like action.* A number of the N-alkyl derivatives in the series of N-(2-chloroethyl)-1-naphthalenemethylamines were tested in dogs to determine whether the depressor effects of acetylcholine were diminished or annulled. The depressor responses to acetylcholine were frequently diminished but never annulled when doses of 5 or 10 mgm./kgm. were injected intravenously. The small diminution noted could have been due, in part, to decreased blood pressure. These findings indicate that the compounds do not exert a moderate or strong atropine-like action. Data in table 3 reveal that doses of 1.0 and 3.0 mgm./kgm. only diminished the mean depressor response to acetylcholine a slight degree; reduction occurred in about one-half of the experiments and therefore could not be closely related to antagonism of epinephrine or histamine which was consistently present. Since antagonism of the depressor action of acetylcholine was an inconstant response and usually not marked in degree it is improbable that these compounds exert an appreciable degree of atropine-like action on the vascular system. Other techniques might reveal an atropine-like action of these compounds on other tissues.

*G. Structure-activity relationships.* The antagonism of epinephrine by 1-naphthalenemethylamine derivatives administered orally to mice was especially pronounced with those compounds in which there was present on the tertiary nitrogen atom an alkyl group of 2 to 4 carbon atoms and a 2-chloroethyl group (table 1; nos. 2 to 8, and 12). When the alkyl group was methyl, activity was low as was found also to be true of the methyl homologue in a series of N-alkyl derivatives of N-(2-chloroethyl)benzhydrylamine (unpublished data). It is unlikely that such low activity with methyl homologues is related to solubility since they are equally or more soluble than higher alkyl homologues which proved more active. It is probable that the apparent decrease in activity of higher alkyl homologues, isobutyl, amyl, and hexyl (nos. 9 to 11), was partly due to decreased solubility. Potency determinations made following intravenous administration would possibly measure inherent activity more accurately. The methoxyethyl homologue exhibited activity equal to that of the n-butyl and sec.-butyl homologues. Some decrease in activity resulted when the ethyl group was substituted by a 2-chloroethyl group (compare 2 and 13).

Activity was diminished or lost even in the presence of an alkyl group if the 2-chloroethyl group was altered or substituted. The chloropropyl derivative (no. 14) exhibited only low activity. No activity was demonstrated with doses of 50 mgm./kgm. when the 2-chloroethyl group was replaced by 2-hydroxyethyl (no. 15).

Two examples are cited which indicate that substitution in the 1-position of the naphthalene ring is important to activity. Activity was not demonstrated in mice with doses of 25 mgm./kgm., with those compounds in which the (2-chloroethyl)ethylaminomethyl group was in the 2-position of the naphthalene ring with unsaturation (no. 16) or partial saturation (no. 17). The compound with chlorine substituted in the 4-position exhibited weak activity in mice and a dose of 10.0 mgm./kgm. intravenously induced epinephrine reversal and antagonized histamine in the one experiment made in a dog.

The antagonism of the bronchoconstrictive action of histamine by 1-naph-

thalenemethylamine derivatives administered subcutaneously to guinea pigs was greatest with the lower alkyl homologues which contained a 2-chloroethyl or 2-bromoethyl group (nos. 1 to 3), the ethyl homologues being the most active. Diminution of histamine-induced bronchospasm was progressively lessened as additional carbon atoms were added to the alkyl groups, i.e., compounds 4 to 11. It would seem unlikely that the apparent progressive decrease in activity was due to failure of adequate absorption from subcutaneous sites of injection. Table 3 indicates that the intravenous injection of the n-butyl and n-hexyl homologues in dogs resulted in an inhibition of the depressor action of histamine which was about as prominent as that effected by the ethyl-2-bromoethyl homologue (no. 3) which proved so highly effective against the bronchoconstrictive action of histamine in guinea pigs. We believe it quite probable that this difference may be due to the fact that the higher alkyl homologues have a selective antihistamine action which prevents relaxation of vascular smooth muscle or capillary dilatation.

Antihistamine activity was lost even in the presence of an alkyl group if the 2-chloroethyl group was replaced by 2-hydroxyethyl (no. 15). The bis-2-chloroethyl compound (no. 13) was weakly active. Substitution of chlorine in the 4-position of the naphthalene ring (no. 19) decreased antihistamine action (compare with nos. 2 and 3) since prevention of bronchospasm was not demonstrated, although the depressor response to histamine was halved in a single dog which received 10.0 mgm./kgm. of the compound.

**DISCUSSION.** These data indicate clearly that the lower alkyl homologues in the series of N-(2-haloalkyl)-1-naphthalenemethylamines block certain actions of both histamine and epinephrine. Such dual action is the more remarkable because of the potency, early onset, and long duration of action demonstrated. The revelation that a single synthetic compound can block effects of histamine and epinephrine, which are in themselves physiologically antagonistic, undoubtedly will stimulate and assist further studies directed toward elucidation of the mode of drug action.

The blocking action of these N-(2-haloalkyl)-1-naphthalenemethylamines is not solely directed against histamine and epinephrine since Wells and Rall (12) have reported that N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine·HBr (compound no. 3 or SY-28) blocks or reverses the pressor action of numerous epinephrine congeners. It is also of interest that it was stated to block the pressor action of 2-(1-naphthylmethyl)imidazoline·HCl (Privine·HCl). Thus, one naphthylmethyl derivative blocked the pressor action of another naphthylmethyl derivative, Privine, and one wonders whether it is purely coincidence that the latter possesses an imidazole ring which occurs also in histamine.

Since various 2-halogenated ethylamines have been demonstrated to block certain effects of histamine, epinephrine and congeners, and Privine, it becomes highly probable that blockade of other agents may be demonstrated with these compounds. Furthermore, synthesis of numerous chemically related compounds will probably provide compounds with other types and combinations of pharmacological activity such as antispasmodic, local anesthetic, quinidine-like, parasympathetic blocking action, etc. A wide spectrum of activity seems possible

in view of the fact that nitrogen mustards and related compounds have a strong affinity for numerous chemical groups of cellular constituents (13, 14, 15).

As indicated in preliminary reports, we have found that N-alkyl-N-(2-chloroethyl)benzhydrylamines were practically devoid of antihistamine action but, like Dibenamine, exerted a moderate degree of adrenergic blocking action (6, 11). N-alkyl-2-(2-biphenyloxy)-2'-diethylamines (2, 5, 6, 10) exerted a moderate degree of antihistamine action and stronger adrenergic blocking action than that exhibited by Dibenamine and the benzhydryl derivatives. Antagonism of both histamine and epinephrine was especially prominent with several 1-naphthalenemethylamines herein described. No strong atropine-like action has been demonstrated with any compound in the three series investigated.

The article which follows concerns the effects of two selected compounds on pressor responses to circulating epinephrine and excitation of adrenergic nerves (10).

#### SUMMARY

Alkyl homologues in a series of N-(2-chloroethyl)-1-naphthalenemethylamines exhibited the remarkable dual property of strongly blocking certain effects of both epinephrine and its physiological antagonist, histamine. The lower alkyl homologues were effective in reducing the toxicity of epinephrine in mice, inducing epinephrine reversal in dogs, reducing toxicity of histamine-aerosol and histamine released during anaphylaxis in guinea pigs, and diminishing depressor responses to injected histamine in dogs.

The most effective compounds were N-(2-chloroethyl)-N-ethyl-1-naphthalene-methylamine (SY-14) and the bromo-analogue (SY-28) which, on a molecular basis were of equal effectiveness in preventing histamine-induced bronchospasm in guinea pigs and antagonizing epinephrine toxicity in mice. Epinephrine reversal and diminished depressor responses to histamine occurred almost immediately after intravenous injection in dogs and effects were of long duration.

Structure-action relationships are discussed and attention is drawn to similar pharmacological properties of related 2-chloroethylamines under investigation.

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# ADRENERGIC BLOCKING DRUGS: III. EFFECTS OF TWO HALOGENATED ETHYLAMINES ON PRESSOR RESPONSES TO EPINEPHRINE, NICOTINE AND ADRENERGIC NERVOUS REFLEXES<sup>1</sup>

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Evidence has been presented (1) that Dibenamine (N-(2-chloroethyl)diethylamine·HCl) induces "epinephrine reversal" and exerts adrenergic blocking action. A preliminary report (2) indicates that similar action is exerted by several N-alkyl-N-(2-chloroethyl)benzhydrylamines. Studies with other substituted 2-halogenated ethylamines not only revealed evidence of adrenergic blocking activity but also an antagonism to histamine (2-8).

In the present study a representative of each of two chemical series of halogenated ethylamines was investigated to determine the ability to block adrenergic reflex mechanisms. N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine·HBr (SY-28) was selected for study because of marked potency with respect to antagonism of both histamine and epinephrine (7), a combination of effects which is unparalleled by any other known compound. N-[2-(2-biphenyloxy)ethyl]-(2-chloroethyl)butylamine·HCl (SY-30) was selected from a series of alkyl homologues since it antagonized epinephrine to an appreciable degree (4) but exerted only weak antagonism of histamine (8). A preliminary report concerning the adrenergic blocking action of these compounds has been published (9).

The chemical structure of the compounds studied is shown along with that of Dibenamine in figure 1. All of these compounds produce irritation at the site of subcutaneous injection. The new compounds proved more potent than Dibenamine with respect to antagonizing the lethal action of epinephrine in mice (3, 6, 7), and the onset of action is much more rapid, being almost immediate. Once established, blocking action is prolonged with all compounds (1-4, 6).

**METHOD.** A method of study was chosen in which the ability to block the effects of injected epinephrine as well as the ability to block the effects of sympathetic stimulation could be determined. For this purpose adrenergic reflex pressor responses to carotid occlusion and to anoxia were employed. In addition, adrenergic activity, manifested as a rise in blood pressure, was induced by ganglionic stimulation with small doses of nicotine.

Mongrel dogs of both sexes were lightly anesthetized with pentobarbital sodium (25 mgm./kgm. intraperitoneally) and prepared for recording arterial blood pressure from the femoral artery. In some experiments, respiration was recorded by means of a Marey tambour connected to a pneumograph tied securely to the side of the chest. Certain of the

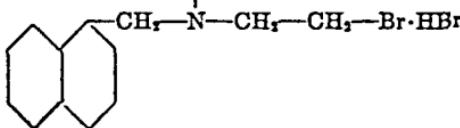
<sup>1</sup> Parke, Davis and Company, Detroit, Michigan supplied a grant in support of this investigation and also the chemical compounds which were synthesized by Drs. G. Rieveschl, Jr., R. W. Fleming and W. R. Coleman.

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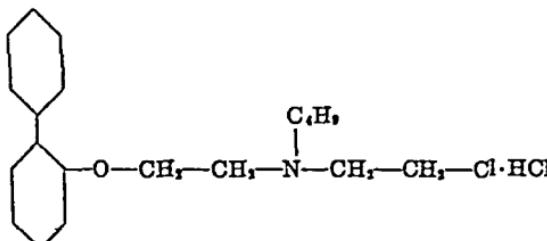
experiments were made in dogs which received 1.0 mgm./kgm. of atropine sulfate (see table 1).

In testing the effects of these adrenergic blocking agents on sympathetic reactivity, the following procedure was employed: (1) epinephrine hydrochloride was injected intravenously in a total dose of 20 gamma, (2) the carotid arteries were occluded below the bifurcation for one minute, (3) anoxic anoxia was induced by rebreathing of nitrogen gas over soda lime for 45 seconds to 2 minutes, depending upon magnitude of the response, and (4) nicotine, in a total dose of 0.15 to 0.8 mgm. was injected intravenously. After repeating

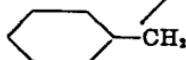
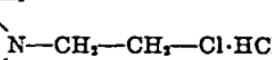
FIG. 1



N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine·HBr  
(SY-28)



N-[2-(2-biphenylyloxy)ethyl]-N-(2-chloroethyl)butylamine·HCl  
(SY-30)



N-(2-chloroethyl)dibenzylamine·HCl

Dibenamine

each of the above procedures twice, SY-30 in a dose of 5.0 mgm./kgm. or SY-28 in a dose of 1.0 mgm./kgm. was administered intravenously over a period of 2 minutes. The above procedures were then carried out again at 10, 35 and 90 minutes after administration of the blocking agent, and the responses compared with those obtained prior to treatment. Figure 2 shows a partial tracing from one experiment illustrating, in part, the method employed. The responses before and after the drug shown in the tracing portray the mean responses obtained as indicated numerically in table 1.

The blocking agents studied are bitter, white crystalline compounds which deteriorate in solution on standing or heating, and hence were freshly prepared before use. Slightly opalescent solutions of SY-28 in concentrations of 0.1 to 0.2 per cent were employed in this

study. Two per cent solutions of SY-30 were made by addition of a few drops of dilute hydrochloric acid.

**RESULTS.** A. *Effect of the blocking agents on the pressor response to epinephrine.* The data in table 1 reveal that these new compounds markedly altered the pressor response to epinephrine. While only depressor effects appear in the

TABLE 1

*Alterations of pressor responses to injected epinephrine, ganglionic stimulation with nicotine and adrenergic nervous reflexes*

PROCEDURE		SY-30† 5.0 MG.M./KG.M., I.V.		SY-28‡ 1.0 MG.M./KG.M., I.V.		UNTREATED CONTROLS	
		Unatropinized	Atropinized	Atropinized	Unatropinized	Atropinized	
No. of dogs.....		5	5	7	4	4	
Epinephrine· HCl; 0.02 mgm.; Total dose	Mean re- sponse,* mm. Hg Before After	+54.0 -23.8§	+42.8 -16.8§	+51.6 -36.4§	+39.7 +35.7	+47.0 +43.5	
	Mean differ- ence ± Standard Error, mm. Hg P¶	77.8 ± 7.9	59.6 ± 9.2	88.0 ± 9.6	4.0 ± 4.0	3.5 ± 3.5	
	P¶	<0.01	<0.01	<0.01	>0.3	>0.3	
Nicotine base #; 0.15-0.8 mgm. Total dose	Mean response, mm. Hg Before After	+23.8 -4.2	+30.2 +1.2	+17.8 -3.2	+15.7 +9.0	+37.2 +35.2	
	Mean differ- ence ± Standard Error, mm. Hg P	27.2 ± 4.2	29.0 ± 3.5	21.0 ± 6.2	6.7 ± 5.5	2.0 ± 2.0	
	P	<0.01	<0.01	<0.01	>0.1	>0.3	
Carotid occlusion	Mean response, mm. Hg Before After	+43.0 +16.0	+33.4 +11.8	+26.1 +14.0	+43.0 +30.7	+34.0 +28.8	
	Mean differ- ence ± Standard Error, mm. Hg P	27.0 ± 5.5	21.6 ± 2.2	12.1 ± 3.8	12.3 ± 6.8	5.2 ± 1.8	
	P	0.01	<0.01	0.02	>0.1	>0.05	

TABLE 1—Concluded

PROCEDURE		SY-30† 5.0 MG/M./KG., I.V.		SY-28‡ 1.0 MG/M./KG., I.V.		UNTREATED CONTROLS	
		Unatropinized	Atropinized	Atropinized	Unatropinized	Atropinized	
		5	5	7	4	4	
Nitrogen inhalation	Mean response, mm. Hg Before	+36.0	+27.8	+24.6	+30.5	+26.2	
	After	-2.0	+2.4	-7.5	+29.5	+23.7	
	Mean difference ± Standard Error, mm. Hg	38.0 ± 8.7	25.4 ± 0.7	32.1 ± 9.1	1.0 ± 1.54	2.5 ± 3.9	
	P	<0.02	<0.01	<0.01	>0.5	>0.5	

\* Mean control responses are the average of two in each of the designated number of animals. Likewise mean test responses are the average of 2, taken at 35 and 90 minutes after treatment, in each of the designated number of animals.

† N-[2-(2-biphenyloxy)ethyl]-N-(2-chloroethyl)butylamine-HCl

‡ N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine-HBr

§ Approximately 50 per cent of these responses were preceded by a small pressor spike; for the statistical analysis only the depressor phase was considered (see text).

¶ Responses to nicotine were occasionally biphasic; above values are based on the pressor phase when this occurred.

¶ Statistically significant when 0.05 or less.

table and were considered for statistical analysis, it should be pointed out that after administration of the blocking agent a biphasic response to epinephrine occurred in approximately 50 per cent of the experiments. This response consisted of a small pressor spike of short duration, followed at once by a longer depressor phase. It is possible that the pressor spike, which averaged only 10 to 12 mm. Hg, was due to increased cardiac output referable to myocardial stimulation which was not blocked by the drugs used. The fact that Dibenamine does not alter the tachycardia induced with epinephrine (1) lends support to this view.

B. *Effect on adrenergic reflex and nicotine pressor responses.* Here the two compounds in their respective doses exerted essentially the same activity. The effect of these adrenergic blocking agents was that of complete blocking of the pressor responses to nicotine and anoxic anoxia, in both atropinized and unatropinized dogs (table 1). Occasionally the drugs caused a definite reversal of the responses. Respiratory stimulation in response to the action of nicotine and anoxic anoxia on chemoreceptors in the carotid body did not appear diminished after treatment with the adrenergic blocking drugs. Furthermore, "epinephrine apnea" was absent and replaced by respiratory stimulation which consistently occurred concurrently with definite depressor responses to epinephrine. The persistence of respiratory reflex responses renders it unlikely that the drugs

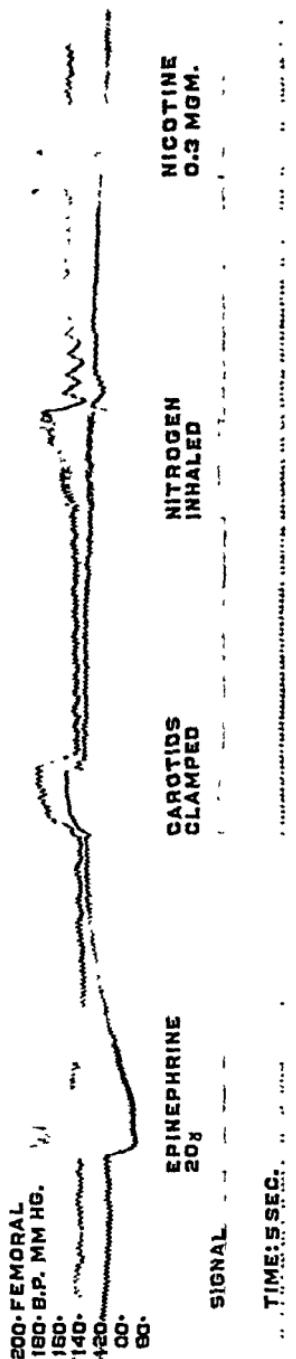


FIG. 2. EFFECT OF ADRENERGIC BLOCKADE ON VARIOUS PRESSOR RESPONSES IN THE PENTOBARBITALIZED DOG  
Upper tracing shows the responses to the various stimuli as indicated.  
Lower tracing shows the responses to the same stimuli 10 minutes after intravenous injection of SY-30 in a dose of 5.0 mgm./kgm.

altered the sensitivity of the receptors or centers in the central nervous system concerned with the respiratory reflexes.

A statistically significant diminution of the carotid sinus pressor reflex was observed. This is in contrast with the complete blocking effect on the anoxic or nicotine pressor response. This diminution, instead of complete blocking, could possibly be due to the fact that carotid occlusion did not cause as much epinephrine to be released from the adrenal medulla as did the anoxic state and injection of nicotine.

C. *Effect on mean arterial blood pressure.* While the effects of the above adrenergic blocking agents were essentially the same on the various pressor responses considered above, the effect of the two agents on mean arterial blood

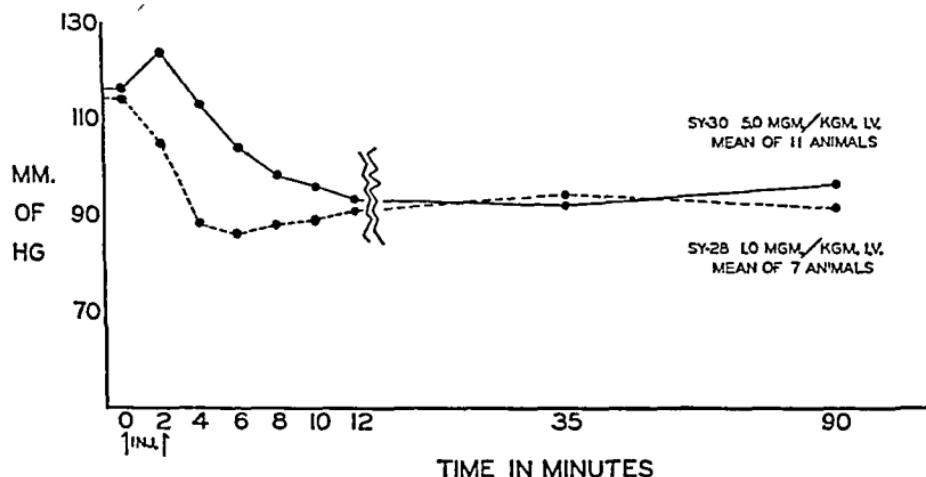


FIG. 3. EFFECT OF ADRENERGIC BLOCKING DRUGS ON MEAN ARTERIAL BLOOD PRESSURE OF THE ANESTHETIZED DOG

pressure differed somewhat. As seen in figure 3, both agents depressed mean arterial blood pressure by 20 to 30 per cent and the effect was slow to subside. This agrees well with the fact that these compounds are long acting; their action from a single dose lasted for 24 to 48 hours or longer (6, 7). SY-28 apparently had a faster onset of action than did the biphenyloxy ethylamine compound. The maximum depression of blood pressure was greatest with SY-28 and occurred within 6 minutes, whereas, with SY-30, maximum hypotension did not occur until after 12 minutes.

DISCUSSION. The results show that SY-28 and SY-30 were capable of reversing epinephrine and blocking or diminishing adrenergic pressor reflexes, as well as the pressor response to small doses of nicotine. This was achieved with SY-28 in relatively small doses—i.e., 1.0 mgm./kgm. intravenously. This is in contrast with other adrenergic blocking agents, with the possible exception of ergotamine (*vide infra*), which require larger doses to produce adrenergic blockade. Thus, 10 to 20 mgm./kgm. of Dibenamine (10) and 3.0 mgm./kgm. of yohimbine (11) were required to diminish or block the pressor sinus reflex.

Priscol, in a dosage of 6.0 to 15.0 mgm./kgm., suppressed the vascular response to carotid occlusion in unilaterally sympathectomized dogs (12). Large doses of the dioxanes, 933 F and 883 F, were also required to produce this effect (13).

Ergotamine, however, in small doses apparently has a selective action in blocking the sinus reflexes. Thus, doses of 0.01 to 0.1 mgm./kgm. of ergotamine tartrate have been shown to be capable of blocking sinus vasomotor reflexes of the cat, but at the same time these doses were unable to block or reverse the action of epinephrine (14, 15). Furthermore, the same doses did not block the blood pressure or respiratory responses to cyanides, nicotine and hypoxia (14). In the cat, larger amounts, in the neighborhood of 1.0 to 2.0 mgm./kgm., were required to diminish or block the excitatory effects of the sympathetic nervous system, as well as reverse the action of epinephrine (14, 15, 16). Von Euler and Schmiederlöw (14) attributed this blocking effect on sinus vasomotor reflexes by small doses of ergotamine to a centrally located selective action. The work of Heymans, *et al.* (17, 18) does not support such a view, however. These workers used the crossed circulation technique and concluded that ergotamine in the cat or dog blocked the vasomotor sinus reflexes by a peripheral action.

Such a selective action as that which likely exists for ergotamine probably does not exist as far as the 2-halogenated ethylamines are concerned. We have observed that if a diminished response to carotid occlusion was obtained, then blocking of the nicotine and anoxic pressor responses and epinephrine reversal were readily demonstrated.

While SY-28, SY-30 and yohimbine (11) blocked the anoxic or asphyxial pressor response, Dibenamine has been reported to reverse this response in cats and dogs (1, 10, 19). A reversal of this response could be expected if sufficient epinephrine were liberated from the adrenal medulla; the amount liberated would probably depend upon the degree of anoxia existing during the various experiments. Under severe degrees of anoxia, epinephrine would be released not only reflexly, but also by a direct action of the anoxic state on the adrenal medulla (20).

SY-28 and SY-30 were observed to cause a moderate decrease of blood pressure. Active adrenergic blocking agents would be expected to cause a depression of mean arterial blood pressure by virtue of diminution or abolition of the influence of sympathetic tone, although a direct vasodilating action of SY-28 and SY-30 has not been ruled out. The degree of depression probably depends in part upon the state of oxygenation existing at the time of injection, since it has been observed in other experiments, where the animal was undoubtedly in a state of hypoxia, that even as small a dose as 0.5 mgm./kgm. of SY-28 infused intravenously over 10 minutes immediately reduced the blood pressure to shock levels.

SY-28 and SY-30 in doses of 1.0 and 5.0 mgm./kgm., respectively, reversed the action of epinephrine but did not block all pressor responses of nervous origin. This appears to be a quantitative and not a qualitative matter and indicates that larger doses would be required to effect complete adrenergic blocking. It is apparent that with all adrenergic blocking agents, the dose

required to effectively block adrenergic nerves (sympatholysis) is several times the dose required to block or diminish responses to injected or circulating epinephrine (adrenolysis). It is therefore not surprising that Dibenamine, in a dose of two to four times that usually employed (10 to 20 mgm./kgm., intravenously) to invoke "epinephrine reversal" failed to block completely certain adrenergic nervous responses (21). The increase in dosage necessary to block adrenergic nervous responses, as compared to dosages needed to block circulating epinephrine, is not peculiar to adrenergic blocking agents since an analogous situation exists with respect to anti-cholinergic drugs. For example, it is well known that small doses of atropine will prevent depressor responses to injected acetylcholine and even block certain cholinergic nervous responses, but it may fail to prevent nervous effects in the urinary bladder and gastro-intestinal tract even though larger doses are administered. It is quite obvious that there is a need for a truly potent and specific adrenergic blocking compound which in tolerated doses will readily block adrenergic nervous effects, particularly if some selective action is exerted on given tissues or organs.

The fact that SY-28 and SY-30 are closely related chemically to Dibenamine at once suggests that the same chemical grouping ( $N-CH_2-CH_2-X$ ) accounts for maximal activity, and that the site of action is probably the same. Nickerson (1, 21) has discussed the site of action of Dibenamine and has come to the conclusion that this agent acts peripherally at the effector cell. While the blocking action of SY-28 and SY-30 on the carotid sinus and anoxic reflex pressor responses could be located centrally and/or in ganglia, it is more likely that such blocking occurred peripherally for the vasomotor and respiratory responses to epinephrine were reversed, and the respiratory accelerating action of anoxia and nicotine were not altered by the compounds. Such a peripheral action would also account for the blocking of the nicotine responses, although a ganglionic site has not been ruled out.

#### SUMMARY

*N*-(2-bromoethyl)-*N*-ethyl-1-naphthalenemethylamine·HBr and *N*-2-(2-biphenyloxy)ethyl-*N*-(2-chloroethyl)butylamine·HCl, in relatively small doses of 1.0 and 5.0 mgm./kgm., intravenously, respectively, have been shown to be active adrenergic blocking compounds by virtue of the ability to reverse the action of epinephrine and to diminish or block the pressor responses to anoxia, carotid occlusion, and small doses of nicotine in dogs. The experimental evidence supports a peripheral adrenergic blocking action.

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# THE FATE OF PROCAINE IN MAN FOLLOWING ITS INTRAVENOUS ADMINISTRATION AND METHODS FOR THE ESTIMATION OF PROCAINE AND DIETHYLAMINOETHANOL<sup>1</sup>

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The local anesthetic, procaine, has been administered intravenously for the control of pain associated with burns, fractures, etc., and in the post-operative period following the trauma of surgical procedures (1, 2, 3). It has also been used for disorders of cardiac rhythm observed during anesthesia (4). Studies were undertaken to identify the transformation products of procaine in the body, and to study the extent to which the activity of the drug is limited by its transformation, or is associated with the derived products. This paper deals with the physiological disposition of procaine and the identification of its derived products.

It has been demonstrated that an enzyme exists in plasma which catalyzes the hydrolysis of the drug to p-aminobenzoic acid and presumably, diethylaminoethanol (5). Little positive information is available, however, concerning the fate of procaine *in vivo*. This is due in no small part to the lack of a suitable method for the determination of procaine and its transformation products in biological tissues.

**CHEMICAL METHODS.** A study of the metabolic fate of procaine required sensitive methods for the estimation of procaine and its metabolic products in biological material.

*Determination of Procaine.* Methods for the estimation of procaine in biological fluids have been previously described. The chief difficulty involved in its estimation is the separation of procaine from its hydrolytic product, p-aminobenzoic acid. The method of Kisch and Strauss separates procaine from p-aminobenzoic acid by an extraction procedure, and then estimates it by the Bratton and Marshall reaction (6). This procedure includes no provision for the inhibition of the enzymatic hydrolysis of the procaine which occurs after the blood is drawn. Moreover, the method is not sensitive enough to measure the small amounts of procaine which occur in plasma during its intravenous administration to man. The method described by Graubard is unsuitable since the measurement includes the large excess of p-aminobenzoic acid derived from the hydrolysis of procaine in the body (7).

The method described below is a modification of that of Kisch and Strauss. The *in vitro* hydrolysis of the procaine is prevented by the addition of sodium arsenite to the blood. The sensitivity of the measurement is considerably enhanced by forming the azo dye in a small volume and assaying its concentration by microspectrophotometry. This permits the estimation of plasma procaine concentrations as low as 100 micrograms per liter.

The procaine is isolated from the biological material by extraction into ethylene di-

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The procaine (novocaine) used in these studies was obtained through the courtesy of the Department of Medical Research, Winthrop-Stearns Inc.

chloride, returned to dilute acid, and then diazotized and coupled with N (1-naphthyl) ethylene diamine. The resulting dye is assayed in a spectrophotometer adapted to small volumes (8).

The specificity of the method was appraised by a distribution technique (9, 10). The results obtained indicate that the substance measured in biological material is identical with authentic procaine.

**Preparation of plasma samples:** Human plasma contains an enzyme which markedly accelerates the hydrolysis of procaine to p-aminobenzoic acid and diethylaminoethanol (5). Procaine in human plasma completely disappears in about two minutes unless the enzymatic activity is inhibited (table 5). The blood is therefore rapidly drawn and immediately placed in a test tube containing sodium arsenite (2 drops of 50 per cent solution per cc. of blood). The contents are mixed by inversion of the tube which has been covered by a microscope slide. The plasma is separated from the cells and analyzed within an hour.

If it is inconvenient to analyze the sample within this time, the plasma proteins may be precipitated with trichloroacetic acid and the procaine estimated in the filtrate. The procaine is stable for at least several days in the filtrate. Aliquots are neutralized with sodium carbonate, buffered to pH 9 and the procaine estimated as described below.

**Procedure.** Add 1 to 5 cc. of plasma or suitably diluted urine (containing up to 5 micrograms of procaine) and 1 cc. of 0.8M borate buffer (pH 9) to 20 cc. of ethylene dichloride<sup>2</sup> in a 60 cc. glass-stoppered bottle. Shake for 10 minutes. Transfer the contents of the bottle to a test tube and centrifuge for 5 minutes. Remove the aqueous phase by aspiration. Transfer 15 cc. of the solvent phase to a 40 cc. conical glass-stoppered tube containing 1 cc. of 1N HCl. Shake for 5 minutes and then centrifuge. Introduce a finely drawn glass tube to the bottom of the lower solvent phase and gently aspirate all but the last traces of the solvent. Add 0.05 cc. of 0.1 per cent sodium nitrite solution. Wait 5 minutes, add 0.05 cc. of 0.5 per cent ammonium sulfamate solution<sup>3</sup>. After 3 minutes add 0.05 cc. of 0.1 per cent N(1-naphthyl) ethylene diamine dihydrochloride. Allow 20 minutes for the color to develop fully. Determine the optical density of the dye at 550 m $\mu$  in the Coleman Model 6 spectrophotometer adapted to microspectrophotometry (8). A reagent blank is run through the procedure. This reagent blank should not give an optical density of more than 0.005 when 1N HCl to which the diazotizing and coupling reagents have been added is used for the zero setting.

Standards are prepared by taking 1 cc. of standard solution and adding nitrite, sulfamate and coupling reagent as described above. A blank of 1N HCl to which the above reagents have been added is used for the zero setting. The optical densities were found to be proportional to concentration. An optical density of about 0.075 was obtained in the adapted Coleman Model 6, when 1 microgram of procaine was run through the procedure. Procaine added to plasma and urine in amounts from 0.5 to 3 micrograms was recovered with adequate precision ( $98 \pm 6$  per cent).

**Determination of Diethylaminoethanol.** A reaction previously described (9), for the estimation of basic organic compounds was used in the estimation of diethylaminoethanol. This reaction involves the formation of a salt of the organic base with methyl orange. This salt is extracted into ethylene dichloride and estimated directly in the solvent. Difficulties were encountered in the estimation of diethylaminoethanol by this method because of the marked water solubility of its methyl orange salt. This solubility was greatly reduced by

<sup>2</sup> A technical grade of ethylene dichloride is purified by successive washings with 1N NaOH, 1N HCl and three washings with water. 1.5 per cent by volume of isoamyl alcohol, reagent grade, which has been similarly treated, is added to the solvent to minimize the adsorption of the compound from the solvent onto the glass surface.

<sup>3</sup> A non-procaine blank, which does not react with the coupling reagent, is extracted from urine. This blank is corrected for by reading the optical density of the solution at this stage before the addition of the coupling reagent and subtracting the reading from the final optical density of the dye.

increasing the concentration of methyl orange, and by reducing the volume of the aqueous methyl orange phase.

The diethylaminoethanol is isolated from biological material by extraction into ethylene dichloride. The ethylene dichloride phase is shaken with methyl orange at pH 5 and the excess methyl orange is removed. The methyl orange, which dissolves in the solvent through salt formation in amounts equivalent to the contained base, is estimated spectrophotometrically.

The specificity of the method was appraised by the distribution technique. The results obtained indicate that the substance measured in the biological material is identical with authentic diethylaminoethanol.

**Procedure.** Add 2 cc. of plasma or suitably diluted sample<sup>4</sup> (containing up to 30 micrograms of diethylaminoethanol), 1 cc. of 1*N* NaOH and 3 grams of potassium iodide<sup>5</sup> to 15 cc. of ethylene dichloride<sup>2</sup> in a 60 cc. glass-stoppered bottle. Shake for 10 minutes. Transfer the contents of the bottle to a test tube and centrifuge for 5 minutes. Transfer as much of the supernatant solvent phase as possible to a 60 cc. glass-stoppered bottle containing 0.1 cc. of methyl orange reagent<sup>6</sup>. Shake for 5 minutes, transfer the contents to a test tube and centrifuge at high speed. Completely remove the supernatant methyl orange layer by aspiration. Pipette 10 cc. of the solvent phase into a colorimeter tube containing 2 cc. of a solution of 2 per cent by volume of sulfuric acid in absolute alcohol. Determine the optical density in a spectrophotometer at 540 m $\mu$ <sup>7</sup>. A reagent blank is run through the same procedure. The reagent blank should not yield an optical density greater than 0.010 when ethylene dichloride plus the alcoholic sulfuric acid is used for zero setting. (Coleman Model 6 spectrophotometer.)

Standards are prepared by taking 2 cc. of standard solution, adding 1 cc. of 1*N* NaOH, 3 grams of potassium iodide and 15 cc. of ethylene dichloride and handling in the same manner as the unknowns. An optical density of about 0.100 is obtained when 5 micrograms of diethylaminoethanol are run through the procedure.

Diethylaminoethanol added to plasma and urine in amounts from 5 to 30 micrograms was recovered with satisfactory precision ( $105 \pm 5$  per cent).

**Procedure for total p-aminobenzoic acid (free and conjugated).** The conjugated forms of p-aminobenzoic acid are deacetylated to p-aminobenzoic and p-aminohippuric acids. The latter compounds are diazotized and coupled with N(1-naphthyl) ethylene diamine. The resulting dyes are assayed at 550 m $\mu$ . The dyes formed from both p-aminobenzoic acid

<sup>4</sup> Organ tissues are prepared for analysis by emulsification in acid as described in a previous paper (10).

<sup>5</sup> The addition of potassium iodide to the aqueous phase makes it heavier than the ethylene dichloride phase. This not only facilitates the handling of the solvent phase, but also decreases the blank to some extent.

<sup>6</sup> The methyl orange reagent used previously in the estimation of organic bases was a saturated solution in 0.5*M* boric acid and contained about 90 mgm. of methyl orange per 100 cc. (9). This solution is too dilute to give a suitable reaction with diethylaminoethanol. However methyl orange is about seven times more soluble in ordinary water. A saturated solution of methyl orange in water when added to a boric acid solution results in a supersaturated solution of methyl orange from which the latter does not crystallize out for some time. Advantage of this phenomenon is taken to produce a relatively high concentration of methyl orange in boric acid solution.

A saturated aqueous solution of the sodium salt of methyl orange is washed several times by shaking with half its volume of ethylene dichloride. The methyl orange reagent is made by diluting this solution with an equal volume of saturated boric acid solution. This dilution is made just prior to using since the methyl orange precipitates out within an hour.

<sup>7</sup> The diethylaminoethanol estimated in plasma includes that derived from the *in vitro* hydrolysis of the procaine also present. The concentration of procaine is usually so small as to involve a negligible correction.

and p-aminohippuric acid yield the same optical density per mole. The estimation may therefore be expressed in terms of total p-aminobenzoic acid. The procaine present is also included in the estimation but is so small that it may be neglected in the calculation of total p-aminobenzoic acid.

The total p-aminobenzoic acid is determined in a filtrate of plasma. To deproteinize the plasma, add 5 cc. of 50 per cent trichloroacetic acid to 2 cc. of plasma and 3 cc. of water. Transfer 5 cc. of the plasma filtrate or diluted urine to a test tube containing 1 cc. of 12*N* HCl. Heat on the water bath for 1 hour. Estimate the resulting aromatic amines by the Bratton and Marshall reaction (11).

*Evidence for the identity of the substances determined in biological fluid.* Conclusions concerning the fate of procaine depend upon a knowledge of the identity of the substances in the biological material. A technique, previously described by us (9, 10), permits the identification and, to a considerable degree, the establishment of purity of a substance being

TABLE I  
*Distribution of procaine and apparent procaine between ethylene dichloride and water at various pH values*

The apparent procaine was obtained by extraction with ethylene dichloride of the plasma of a subject receiving procaine by intravenous infusion. The compound was returned to dilute acid. Aliquots of this solution and an authentic procaine solution were adjusted to various pH values and shaken with equal volumes of ethylene dichloride. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	AUTHENTIC PROCAINE	APPARENT PROCAINE FROM PLASMA
5.6	0.18	0.15
6.3	0.57	0.54
7.0	0.93	0.89

measured. It involves a comparison of the distributions of the substance with those of the authentic substance in a two-phase system consisting of an organic solvent and water at various pH values. Dissimilar distributions indicate the presence of a substance different from the authentic compound. To escape detection, a transformation product would have to have not only a similar dissociation constant but identical solubility characteristics in two solvents.

The examination in the case of procaine and diethylaminoethanol was made with ethylene dichloride extracts of plasma from patients who had received procaine intravenously. The partitions of procaine and diethylaminoethanol between ethylene dichloride and water at various pH values was compared with those of the apparent compounds from the biological fluids. The results with each substance showed that, within experimental error, the apparent and the authentic compound had the same solubility characteristics and were therefore presumably the same compound (table 1-2).

**FATE OF PROCAINE IN MAN. *Procaine and its transformation products found in urine after procaine administration.*** Information concerning the metabolic products of procaine was obtained from the urine of 5 subjects given 2 grams of the drug by intravenous infusion. The infusion time varied from 45 to 125 minutes. The urines were collected over a period of 24 hours. The excretion of the various transformation products subsequent to this time was negligible. Approximately 2 per cent of the administered procaine was excreted unchanged, indicating that the drug is almost completely metabolized in the body. p-

Aminobenzoic acid and its various conjugates found in the urine<sup>8</sup> were equivalent to about 80 per cent, while the diethylaminoethanol was equivalent to about 30 per cent of the administered procaine (table 3).

*Fate of diethylaminoethanol and p-aminobenzoic acid in the body.* Further information concerning the fate of procaine was obtained by studying the fate of its metabolites. A combined dose of 1 gram of diethylaminoethanol and 1

TABLE 2

*Distribution of diethylaminoethanol and apparent diethylaminoethanol between ethylene dichloride and water at various pH values*

The apparent diethylaminoethanol was obtained by extraction with ethylene dichloride of the plasma of a subject receiving procaine. Aliquots of this solution and of an ethylene dichloride solution of authentic diethylaminoethanol were shaken with  $\frac{1}{4}$  volume of water at various pH values. The fraction of the compound extracted at various pH values is expressed as the ratio of the compound in the organic phase to total compound.

pH	AUTHENTIC DIETHYLAMINOETHANOL	APPARENT DIETHYLAMINOETHANOL FROM PLASMA
8	0.16	0.12
9	0.52	0.52
10	0.93	0.95
11	1.00	0.99

TABLE 3

*The metabolic fate of procaine in man*

Recovery of procaine and its metabolic products from the urine of subjects after the intravenous administration of 2 grams of procaine.

The urine was collected over a period of 24 hours. The proportion of the various metabolites is expressed in percentage of the theoretical amounts that could occur from the amount of procaine administered.

PROCaine	DIETHYLAMINOETHANOL	P-AMINOBENZOIC ACID FREE AND CONJUGATED
per cent	per cent	per cent
1.4	26	75
1.2	33	98
2.3	37	78
1.0	21	64
2.7	28	80

gram of p-aminobenzoic acid was administered intravenously in 250 cc. volume to 2 subjects. Urine was collected over a period of 24 hours. About 33 per cent of the administered dose of diethylaminoethanol was excreted unchanged. About 90 per cent of the p-aminobenzoic acid was recovered in the urine in the form of p-aminobenzoic acid and its various conjugates.

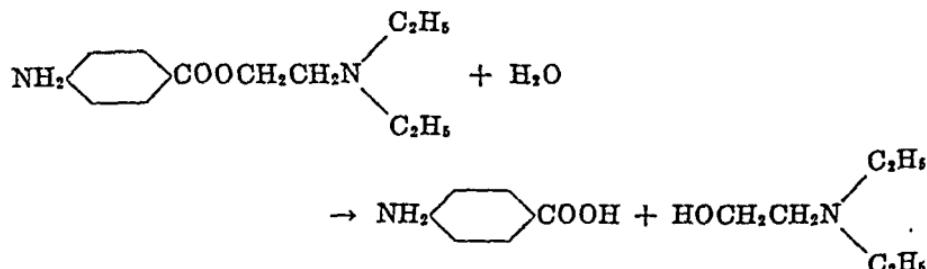
\* The identity of the various p-aminobenzoic acid conjugates was not determined since this information was not relevant to the present problem. These conjugates have been reputed to be predominantly free and acetylated p-aminobenzoic acid.

The above results indicate that about the same fraction of p-aminobenzoic acid and diethylaminoethanol are excreted whether they are given directly or combined as procaine. This suggests that the main, if not the only, route of

TABLE 4  
*Plasma levels of procaine and its metabolites during and after the intravenous infusion of 2 grams to man*

TIME	PROCaine	DIETHYLAMINOETHANOL	TOTAL P-AMINOBENZOIC ACID FREE AND CONJUGATED
Subject A			
minutes after start of infusion	mgm./L.	mgm./L.	mgm./L.
15	0.1	1.3	2
35	0.2	2.2	6.3
75	0.2	2.6	10.6
105	0.3	2.8	12.9
minutes after end of infusion			
15	0.2	2.7	13
35	0.1	2.2	11.5
60	0.0	1.7	8.2
Subject B			
minutes after start of infusion	mgm./L.	mgm./L.	mgm./L.
20	0.2	2.0	4.1
40	0.3	3.6	9.4
107	0.35	3.7	15.1
minutes after end of infusion			
20	0.2	3.2	11.5

metabolism of procaine is hydrolytic cleavage to p-aminobenzoic acid and diethylaminoethanol as follows:



The diethylaminoethanol is further metabolized but the nature of this transformation is not yet known.

*Procaine and its transformation products found in plasma.* Two grams of procaine were administered to 5 subjects by intravenous infusion over periods of 45 to 125 minutes. The concentrations of procaine in plasma were consistently low during the infusion (table 4—2 typical experiments). The diethylamino-

ethanol and total p-aminobenzoic acid levels on the other hand were relatively high and rose steadily during the time of the infusion. At the termination of the infusion, the plasma levels of procaine soon were negligible, whereas those of diethylaminoethanol and total p-aminobenzoic acid persisted for some time. These results indicate that procaine, after its intravenous injection, is hydrolyzed in the body at an unusually rapid rate.

*The role of plasma in the breakdown of procaine.* The extent to which the enzyme in plasma is responsible for the breakdown of procaine was investigated. Procaine was added to fresh human plasma at 37°C to a concentration of 5 micrograms per cc. Samples for procaine analysis were taken in 1, 2, 3, and 6 minutes. It is evident from the results that procaine was hydrolyzed extremely rapidly in plasma *in vitro* (table 5). Eighty to 100 per cent of the procaine broke down within 2 minutes (4 experiments). The derived products were identified as p-aminobenzoic acid and diethylaminoethanol by means of the distribution technique as described under methods.

TABLE 5

*The in vitro hydrolysis of procaine in human plasma at 37°C.*

One-tenth cc. of 0.02 per cent procaine solution was added to 4 cc. of plasma. The extent of hydrolysis is expressed in percentage of the amount of procaine added to the plasma.

TIME	PLASMA A per cent	PLASMA B per cent	PLASMA C per cent	PLASMA D per cent
minutes				
1	55	89	72	83
2	80	100	87	97
3	91	100	96	99
6	100	100	100	100

It is generally considered that the liver is the major site for the destruction of local anesthetics, including procaine (12). These conclusions were derived from experiments in which the drugs lost their toxicity after perfusion through animal livers. Also, animals with damaged livers were found to be more susceptible to the toxic actions of local anesthetics (12). On this basis, it has been suggested that their extensive use should perhaps be avoided in patients with severely diseased livers. The results reported here suggest that the liver is not an important site for the transformation of procaine, at least in the human. The intravenous infusion of procaine at a rate of 20 mgm. per minute resulted in plasma levels of about 0.2 mgm. per liter as noted above. The rate of transformation of procaine by the body may be assumed to equal the infusion rate since the urinary excretion of procaine is negligible. Assuming that the hepatic plasma flow is a liter per minute and that the plasma is completely cleared of procaine at each passage of blood through the liver, the liver could hydrolyze only 0.2 mgm. of the drug each minute. Since 20 mgm. of procaine are being hydrolyzed per minute in the body, the compound must be transformed mainly by extra-hepatic mechanisms. The rapid rate of hydrolysis of the procaine in plasma *in vitro* shown above, suggests the plasma to be the main site of procaine

break-down. The major factor in determining the safety of a particular local anesthetic drug may well be the speed at which it is hydrolyzed by plasma.

Preliminary work indicates that local anesthetics including procaine are more stable in dog plasma than in human. It is possible that in some species, the liver may play a more important role in the hydrolysis of these esters.

DISCUSSION. Diethylaminoethanol possesses a trialkylamino group. This group is also present in procaine and many other compounds which possess in common some or all of the following pharmacological properties: local anesthesia, quinidine-like action on the heart, spasmolytic action on smooth muscle, analgesia, and anti-allergic action. Examples of other compounds that also have most of these properties are demerol, benadryl and quinidine. The extreme rapidity with which procaine disappears from the plasma and the relative persistence and high concentration of diethylaminoethanol, suggests that the latter might account for some of the pharmacological properties ascribed to the parent drug. It therefore seems desirable to compare its properties with those of procaine. These studies are now in progress.

#### SUMMARY

1. Methods are described for the estimation of procaine and its transformation products in biological fluids and tissues *in vivo*.
2. The first step in the metabolism of procaine after its intravenous administration to man was shown to be hydrolysis to diethylaminoethanol and p-aminobenzoic acid. The hydrolysis takes place with unusual rapidity. Diethylaminoethanol is further metabolized in large part, but the nature of its transformation is not known.
3. The hydrolysis of procaine occurs mainly in the plasma.

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# PROTECTIVE ACTION OF COMBINATIONS OF DIPHENHYDRAMINE AND AMINOPHYLLINE IN FATAL BRONCHOSPASM IN GUINEA PIGS DUE TO MECHOLYL AEROSOL

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Aminophylline is one of the most valuable remedies used in the treatment of bronchial asthma in man (1). Diphenhydramine, on the other hand, like the other antihistaminic drugs, has proved relatively ineffective in this condition. Recently Segal *et al.* (2) reported that the intravenous injection into asthmatic persons of histamine or acetyl- $\beta$ -methylcholine chloride (mecholyl) produces a marked diminution in vital capacity. Some patients state that mecholyl produces symptoms more nearly resembling their spontaneous asthmatic attacks than does histamine (3). These observations of Segal and his associates suggest that mecholyl bronchospasm may be used as a laboratory tool to investigate the potential anti-asthmatic action of drugs. In this laboratory aminophylline and diphenhydramine have each been found effective in protecting guinea pigs from fatal bronchospasm induced by mecholyl aerosol.

This is the report of an investigation into the nature of the protective action of combinations of aminophylline and diphenhydramine against such fatal bronchospasm in the guinea pig. The report also includes the acute intraperitoneal toxicity of these combinations in the same species.

The method of Loew, Kaiser and Moore (4) for studying protection against fatality induced in guinea pigs by a histamine spray, has been adapted for this study by substituting a 2.4 per cent solution of mecholyl for histamine. Guinea pigs react to the mecholyl vapor in a fashion very similar to their response to histamine with a mortality consistently near 100 per cent. This concentration (2.4 per cent) has, therefore, been used in all the experiments described below.

In addition to diphenhydramine and aminophylline used individually, the two drugs have been combined in three ratios: one weight of diphenhydramine with one, two and four weights, respectively, of aminophylline. For the convenience of water-solubility, the hydrochloride salt of diphenhydramine was used, but dosages are reported in terms of the base. Mixtures were prepared immediately before use by combining clear solutions of the two drugs. This produces a milky suspension which does not settle for many hours. These five preparations were administered intraperitoneally fifteen minutes before exposing the guinea pigs to the mecholyl aerosol. Similar preparations were used for determining acute intraperitoneal toxicity.

Death from the mecholyl aerosol regularly occurs within 15 minutes after introducing the mist; therefore, fatalities in treated animals occurring within this 15-minute interval have been included in the tabulation of results. A few of the highest doses of the "1+4" mixture were toxic *per se* and killed after 45 minutes, an observation also noted for these same doses in the toxicity tests.

For all the studies, guinea pigs of both sexes weighing between 230 and 300 grams were used. They were obtained from a single dealer as thoroughly mixed stock from several of his regular breeders.

Protection against mecholyl bronchospasm was generally determined on sixteen guinea pigs for each dose, except in three instances (noted in table 1) where larger numbers were used. The dosages were based on a logarithmic scale to facilitate later statistical analysis. Table 1 gives the range of these doses together with their resulting percentage protections.

The toxicity studies were conducted on two separate groups of otherwise untreated guinea pigs. Deaths observed up to about 54 hours are reported. A few deaths occurring after this time were excluded because they were isolated and seemed more like the occasional "spontaneous" deaths which occur regularly among stock pigs in the laboratory. The data for the two series were consistent and have been pooled for tabulation in table 2.

**ANALYSIS OF RESULTS.** The method for evaluating dose-effect data recently described by Wilcoxon and Litchfield (5) has been used. The horizontal rows

TABLE 1

*Protection from mecholyl aerosol by various dosage-combinations of diphenhydramine and aminophylline*

(All doses in mgm. per kgm. on 16 guinea pigs, except as noted)

RATIO OF D + A	DOSE (D + A)* % PROTECTION	DOSE (D + A) % PROTECTION				
1 + 0	6 0%	9 6%	13.5 6%	20 19%	30 25%	45† 90%
1 + 1	12 12%	18 0%	27 6%	40 44%	60 69%	
1 + 2	18 0%	27 6%	40.5 37%	60‡ 50%	90 81%	
1 + 4	30 12%	45 25%	67.5‡ 64%	100 44%	150 81%	
0 + 1	24 0%	36 6%	54 25%	80 12%	120 69%	

\* D = Diphenhydramine; A = Aminophylline.

† 20 guinea pigs.

‡ 36 guinea pigs.

in table 1 give the per cent protection obtained with the doses of each of the five ratios of diphenhydramine and aminophylline. When these per cent protections are plotted against their respective dosages (D+A) on logarithmic-probability paper, each series of points is fitted with a straight line whose goodness of fit, for a probability of 0.05, is verified by the Chi-square test. Toxicity data have been evaluated in the same manner. The experimental ED<sub>50</sub> and LD<sub>50</sub> values, the slope of the lines from which these are obtained, and the factors for calculating their respective parameters are given in table 3. The lines do not diverge significantly from parallelism in either the case of the protection or the case of the toxicity data.

Bliss (6) has suggested that drugs with parallel dosage-response curves, such as those described above, may have additive combined actions. To test this hypothesis, use has been made of the ED<sub>50</sub> and LD<sub>50</sub> data in table 3. When each

TABLE 2  
Acute intraperitoneal toxicity of various dosage-combinations of diphenhydramine and aminophylline\*

TOTAL DOSED + A MG.M./KG.M.	RATIO OF D + A				
	1+0	1+1	1+2	1+4	0+1
40		0/6			
45	0/6				
60	1/10	2/6 0/10	0/6		
70	0/6			0/6	
80	5/10	4/10	0/10	0/10	
90		4/6	0/6		
100	4/6			2/6	
110	9/10	6/10	2/10	0/10	0/10
140		4/6	4/6		
150				3/6	2/6
155	6/10	8/10	7/10	5/10	4/10
200		5/6			
210			6/6 9/10	10/10	9/10
230				5/6	5/6
295					10/10
350					6/6
450				6/6	

\* Six guinea pigs were used for each dose in the first experiment and ten for each dose in the second experiment.

TABLE 3

Calculated data on therapeutic and toxic actions of diphenhydramine and aminophylline combinations

RATIO OF D + A*	MECHOLYL AEROSOL TESTS				INTRAPERITONEAL TOXICITY			
	ED <sub>50</sub> † mgm./kgm.	fED <sub>50</sub> †	Slope	fSlope †	LD <sub>50</sub> † mgm./kgm.	fLD <sub>50</sub> †	Slope	fSlope †
1 + 0	33.5 (33.5 + 0)	1.33	2.05	1.40	82.5 (82.5 + 0)	1.17	1.31	1.17
1 + 1	46.2 (23.1 + 23.1)	1.30	1.69	1.30	102 (51.0 + 51.0)	1.22	1.46	1.25
1 + 2	56.5 (18.8 + 37.7)	1.19	1.69	1.21	131 (43.7 + 87.3)	1.16	1.31	1.16
1 + 4	77.0 (15.4 + 61.6)	1.26	2.00	1.28	154 (30.8 + 123.2)	1.23	1.35	1.21
0 + 1	104 (0 + 104)	1.28	1.85	1.35	172 (0 + 172)	1.17	1.33	1.15

\* D = Diphenhydramine; A = Aminophylline.

† In each entry the upper number is the sum of D + A and the pair of numbers in parentheses are the individual D and A values.

‡ The "f" of a term is the factor by which that term is multiplied and divided to determine its confidence limits at P = 0.05.

diphenhydramine component is plotted against its respective aminophylline component, two series of five points are obtained to which lines can be fitted closely by the method of least squares. These two lines, with their respective equations, are shown in figure 1. The close agreement of the data to the lines is apparent.

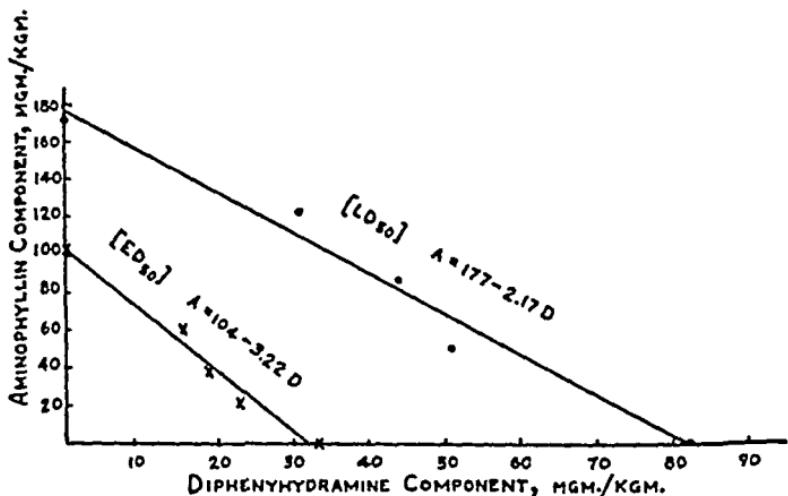


FIG. 1. RELATIONSHIP OF DIPHENHYDRAMINE AND AMINOPHYLLINE COMPONENTS IN THE ED<sub>50</sub> AND LD<sub>50</sub> DOSES FOR THE VARIOUS MIXTURES

These equations in figure 1 give constants which equate the doses of diphenhydramine and aminophylline for equal effects. In other words,

$$1 \text{ gram diphenhydramine} \equiv 3.22 \text{ grams aminophylline}$$

for equivalent protection of guinea pigs from bronchospasm due to mecholyl aerosol; and

$$1 \text{ gram diphenhydramine} \equiv 2.17 \text{ grams aminophylline}$$

for equivalent mortality from intraperitoneal injection in guinea pigs. Any dose of diphenhydramine alone or in combination with aminophylline can, therefore, be converted into terms of aminophylline (and *vice versa*). When the original doses are so converted into terms of aminophylline and these equivalent doses are plotted against the responses (6), the resulting curves do not differ significantly from the original dosage-response curves for aminophylline. Chen (7) in similar experiments using epinephrine and diphenhydramine found that the joint protective effects against histamine spray are additive while the toxic effects at low dosages of diphenhydramine are "synergistic in nature." In the present experiments, both the protection from mecholyl aerosol and the toxicity data indicate simple addition for diphenhydramine and aminophylline.

**SUMMARY AND CONCLUSIONS**

1. Diphenhydramine and aminophylline, alone and in combination, are effective in preventing fatal bronchospasm induced in guinea pigs by a mecholyl aerosol.
2. When used in combination, the protective and intraperitoneal lethal actions are additive in nature.
3. For protection, 1 gram of diphenhydramine is equivalent to 3.22 grams of aminophylline. In the case of intraperitoneal toxicity, 1 gram of diphenhydramine is equivalent to 2.17 grams of aminophylline.

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# ANTAGONISM OF RUTIN TO CAPILLARY EFFECTS OF SODIUM BISULFITE AND PROCAINE

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One of the chief difficulties in the study of the physiological and pharmacological action of rutin and other compounds presumed to produce specific effects upon certain capillary properties is the lack of satisfactory methods to demonstrate such effects. Recently Griffith and associates (1) reported that rutin hastened recovery from irradiation injuries in experimental animals. Ambrose and DeEds (2) demonstrated that large doses of rutin reduced the capillary permeability caused by local application of chloroform or injection of histamine as determined by the trypan blue method. Wilson and co-workers (3, 4) claim a slight decrease of histamine toxicity in guinea pigs by pretreatment with rutin. Raiman et al. (5) reported that rutin treatment greatly reduced the fatality rate due to anaphylactic shock in guinea pigs; however Roth and Shepperd (6) in this laboratory found only slight, if any, protective effect of rutin against anaphylactic shock, and none against the LD<sub>100</sub> of histamine.

This publication concerns itself with studies of the action of rutin upon the capillaries by another procedure which permits a more quantitative evaluation of its effect.

Richards (7) reported that sodium bisulfite (NaHSO<sub>3</sub>) increased the toxicity of epinephrine if these two compounds were injected simultaneously at the same site subcutaneously or intramuscularly. No increase of toxicity was noted when the drugs were injected at separate places or if they were given intravenously. He concluded that sodium bisulfite exerts a specific effect upon the capillaries which permits epinephrine to enter the blood stream at an accelerated rate. These observations have been confirmed by others (8, 9).

Richards et al. have shown that this action of the bisulfite ion is not confined to epinephrine but is present also with other drugs, as for instance procaine (10, 11). Increased capillary permeability caused by sodium bisulfite was demonstrated by Tainter (12) with the trypan blue method.

Rats of both sexes usually weighing between 120 and 170 grams were used. Epinephrine base was dissolved according to the instructions of USP XIII. Such solutions, containing 1.0 mgm. epinephrine base per cc., with or without the addition of 2 mgm. sodium bisulfite per cc., were injected intramuscularly into the hind legs of rats. Rutin solutions were prepared freshly daily. One gram of rutin powder was suspended in some water and 0.69 gram methylglucamine base was added. The mixture was diluted up to 10 cc., slightly heated and well shaken. This usually resulted in a complete solution of the rutin at a pH of 7.5 to 8. Unless otherwise indicated rutin was given by slow intravenous injection. The LD<sub>50</sub> of epinephrine hydrochloride in rats was previously found to be 2.56 mgm./kgm. by intramuscular injection (7), but varies considerably with the strain of rats. In the present study the LD<sub>50</sub> was found to be approximately 4 mgm./kgm. In this investigation we preferred to use dose levels which would kill the majority of the animals. A dose of 0.9 mgm./kgm.

epinephrine and 0.2 per cent sodium bisulfite killed 9 out of 10 animals and 1.0 mgm./kgm. was fatal to 19 out of 21 (part A of figure 1). Thus the great difference between the toxic effect of plain epinephrine solutions and those containing sodium bisulfite was confirmed.

Doses of 100 to 125 mgm. rutin per rat, injected intravenously, produced symptoms of weakness and depression. A dose of 75 mgm. or less caused only slight depression. Rats were injected with 50 or 75 mgm. rutin, respectively, into the tail vein. After ten minutes had elapsed epinephrine with sodium bisulfite was administered intramuscularly. The results of these experiments are shown in a graphic manner in figure 1, part A. It is evident that with 50 mgm. rutin a marked reduction of fatalities occurred. An equivalent amount of a methylglucamine hydrochloride solution adjusted to the same pH provided no protection against subsequent administration of such epinephrine sodium bisulfite mixtures.

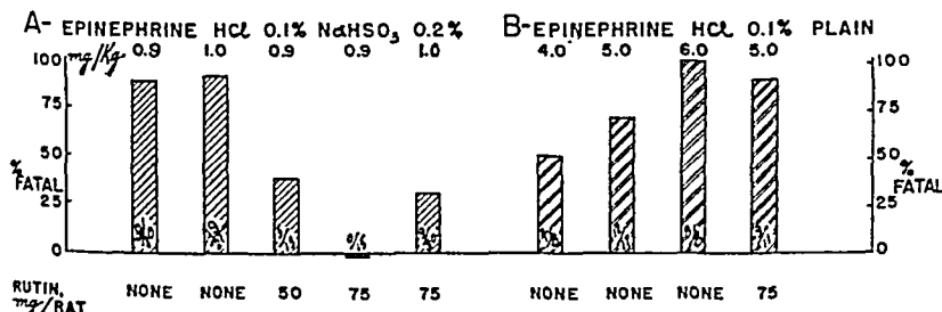


FIG. 1. Epinephrine data refer to the base. Rutin solubilized by methylglucamine given 10 minutes prior to the epinephrine. All injections were made i.m. in rats. Figures within the columns = number fatal/number injected.

It was obviously necessary to investigate the effect of rutin upon the toxicity of an epinephrine solution alone, as shown in part B, figure 1. It can be seen that pretreatment with 75 mgm. rutin intravenously did not reduce, but slightly increased, the fatalities following the injection of 5 mgm./kgm. epinephrine. Thus the action of rutin cannot be explained by a direct effect upon epinephrine toxicity.

It was observed that the urine of rats which had been injected with rutin soon showed the typical rutin color. Other investigators had already pointed out that rutin exerts its effect probably only for a limited period of time. For this reason the duration of action of rutin was studied. Seven groups of 8 to 10 rats each were formed. All were injected with 75 mgm. rutin intravenously and 0.9 mgm./kgm. epinephrine plus 0.2 per cent sodium bisulfite was given intramuscularly after 10, 30, 45, 60 or 100 minutes, respectively, to the separate groups. The results can be seen on figure 2; at the end of one hour the fatalities were about one-half that found without rutin treatment.

In view of the statement in the literature (3) that rutin sensitizes the isolated uterus to the effect of epinephrine and that such action is probably due to a protection of the epinephrine by the rutin, we investigated whether or not a sensi-

tizing effect of rutin towards epinephrine action could be demonstrated by other methods. Perfusion of the isolated frog leg with Ringer solution by the Laewen Trendelenburg method was used. The actions of epinephrine and rutin were studied on three such preparations. One-tenth cc. of epinephrine hydrochloride solutions in concentrations of 1:200,000 to 1:1,000,000 was injected into the rubber tubing just above the cannula and the percentage reduction of the drop rate determined. After several control tests were made the preparation was

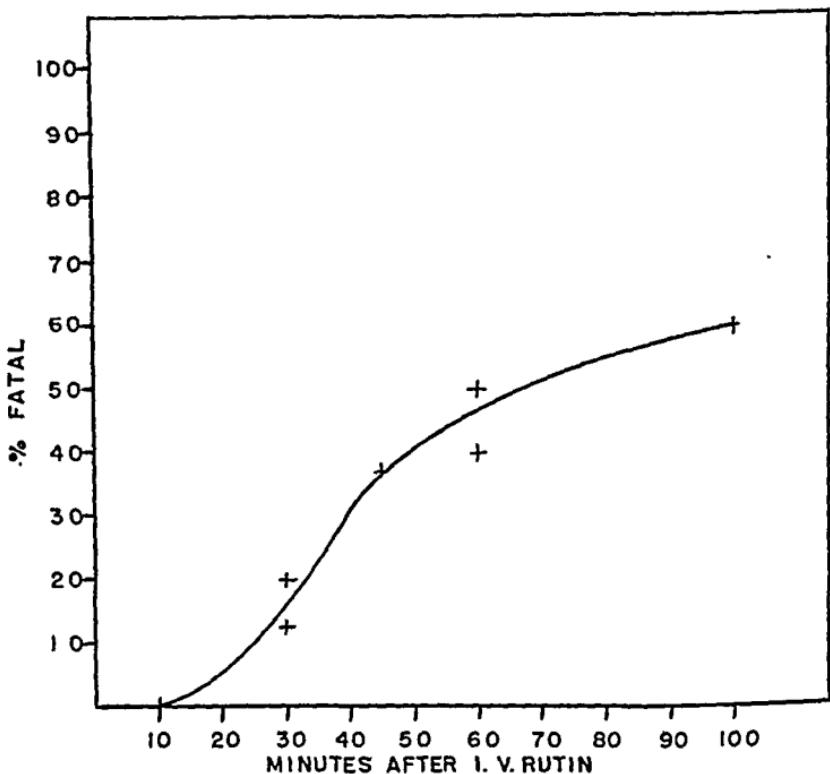


FIG. 2. Seven groups of rats were injected with 75 mgm. rutin i.v. and 0.9 mgm./kgm. epinephrine with 0.2% sodium bisulfite i.m. at stated intervals.

perfused with a Ringer solution containing 0.05 per cent rutin. Higher concentrations of rutin produced a slight vasoconstriction of their own. After this rutin-Ringer solution had perfused for a few minutes the injections with epinephrine were repeated as above. The degree of vasoconstriction during this phase of the experiment was sometimes, but not consistently, greater than before the addition of rutin. Accordingly we did not feel that these experiments could serve as sufficient evidence to show a sensitizing effect of rutin to epinephrine.

In other experiments an attempt was made to demonstrate a sensitizing effect of rutin upon the typical vasopressor action of epinephrine in cats. The rise of blood pressure after injections of small amounts of epinephrine hydrochloride in anesthetized cats was recorded. This was repeated after a constant intra-

venous infusion with a rutin methylglucamine solution (1-11 mgm./kgm./min.) had been started and continued for about 5 minutes. Because the results were equivocal they are not given in detail. Occasionally the reaction to the epinephrine test dose seemed to be slightly (10 to 20 per cent) greater after than before the rutin infusion. However, in other trials no such effect was observed. During infusions with 3-11 mgm./kgm. per min. rutin, a slight rise of blood pressure (about 20 mm. Hg) was sometimes seen.

In a previous investigation (11) it was shown that sodium bisulfite increases the toxicity of intramuscularly injected procaine to an even greater extent than

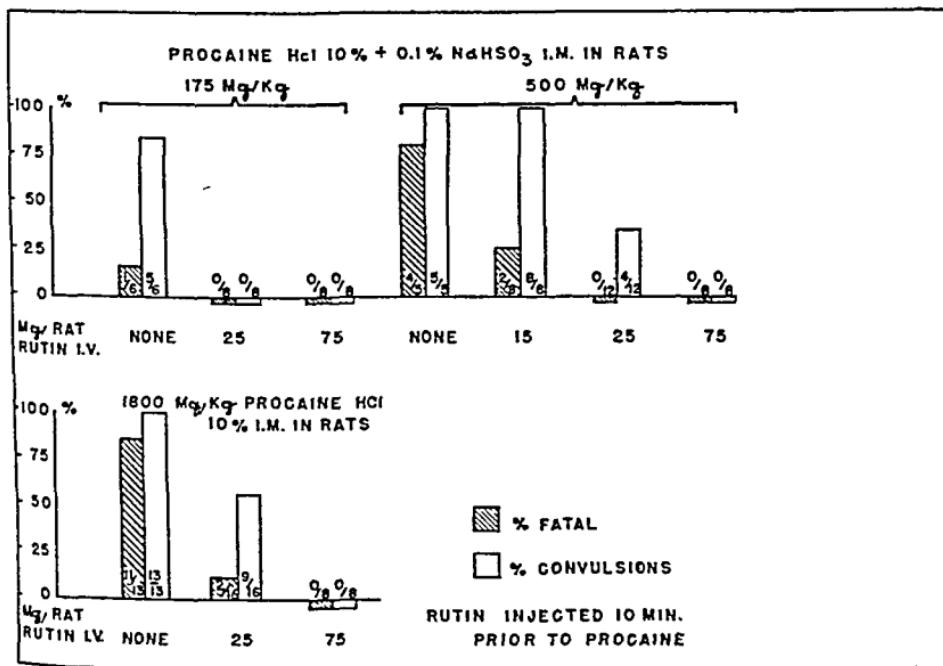


FIG. 3

that of epinephrine. The effect of rutin upon this phenomenon was investigated. Rats were used for these experiments. Injections of 175 mgm./kgm. of procaine with 0.1 per cent sodium bisulfite is convulsive in approximately 80 per cent of the animals, and fatal in about 20 per cent. With 500 mgm./kgm. the incidence of convolution is 100 per cent with 80 per cent fatalities. Rats were injected intravenously with doses of 15, 25, or 75 mgm. rutin, and procaine hydrochloride with the sodium bisulfite was administered intramuscularly ten minutes later. The results are given in figure 3. The left upper quadrant of this figure shows the results obtained against the smaller dose of procaine. Pretreatment with 25 or 75 mgm. rutin reduced both fatal and convulsive rate to 0. In further experiments 10 rats were pretreated with 15 mgm. rutin; none died and 3 convulsed following the procaine injection, indicating a definite protective effect of this dose.

This protective action of rutin against the toxic effects of procaine is even more strikingly shown by the results obtained after the injection of 500 mgm./kgm. procaine with sodium bisulfite. These data are given in the right upper quadrant of figure 3. As little as 15 mgm. rutin reduced the fatality rate from approximately 80 per cent to about 25 per cent, but did not prevent the occurrence of convulsions. When the amount of rutin was increased to 25 mgm. none of the animals died and 33 per cent of them had convulsions. With 75 mgm. both fatalities and convulsions were abolished.

As will be remembered, we had investigated the possibility that rutin might reduce the toxicity of epinephrine alone. Similar experiment with procaine appeared indicated. Procaine hydrochloride given intramuscularly to rats possesses a relatively low toxicity. At a dose of 1800 mgm./kgm. of procaine approximately 85 per cent of the animals die and all experience convulsions. In animals injected with 25 mgm. rutin intravenously 10 minutes prior to this dose of procaine the fatality rate was reduced to approximately 12 per cent and the incidence of convulsions to about 57 per cent (lower figure 2). With 75 mgm. rutin there were no fatalities or convulsions. Thus a difference was noted between the experiments with procaine and corresponding ones with epinephrine, inasmuch as rutin reduced the toxicity of epinephrine with sodium bisulfite but did not affect the toxicity of epinephrine hydrochloride solutions, while the toxic effects of procaine either with or without sodium bisulfite were favorably influenced by pretreatment with rutin.

This observation made it necessary to investigate whether or not the reduction in toxicity of procaine is based upon a systemic effect rather than upon a peripheral action upon the speed of procaine absorption. For this purpose we injected procaine intravenously into rats after rutin pretreatment. It was found that 50 or 75 mgm. rutin did not affect the incidence of convulsions or the fatality rate after intravenous administration of 55 mgm./kgm. procaine, which is the approximate LD<sub>50</sub>. Thus a systemic antagonistic effect of rutin upon procaine toxicity could be excluded.

Because of the results obtained with procaine it appeared desirable to investigate another convulsant drug to study the effect of rutin and sodium bisulfite upon the incidence of convulsions and fatality. Pentamethylene tetrazol (metrazol) was chosen. Each of 12 rats injected intramuscularly with 75 mgm./kgm. metrazol experienced convulsions while 7 of 12, or 58 per cent died. These experiments were then repeated by giving somewhat smaller doses, namely 45 and 60 mgm./kgm. of metrazol mixed with 0.1 per cent sodium bisulfite to six rats each. An effect of sodium bisulfite, as observed with epinephrine and procaine, should result in a high incidence of convulsions and some fatalities with these otherwise non-fatal doses. However, only one of the rats injected with these doses had convulsions; thus it may be concluded that sodium bisulfite does not significantly affect the toxicity of intramuscularly administered metrazol.

Ten rats were injected with 75 mgm. rutin intravenously followed 10 minutes later by 75 mgm./kgm. metrazol intramuscularly. Sixty per cent of these rats had convulsions and 50 per cent died. This does not indicate a statistically sig-

nificant reduction of the toxic effects of metrazol by previous rutin administration.

Reference has been made above to the paper by Ambrose and DeEds (2) who had shown that rutin reduces the capillary permeability following local application of chloroform or intradermal injection of histamine in the skin of white rabbits. We have conducted similar experiments in a modified form. White rabbits of  $1\frac{1}{2}$  to  $2\frac{1}{2}$  kgm. weight were injected with 200 mgm. per animal rutin in methyl-glucamine solution followed 15 minutes later by 5 cc. of a 1 per cent trypan blue solution intravenously. We noticed frequently that the general bluish discoloration of untreated rabbits following the trypan blue injection was noticeably stronger than in those pretreated with rutin. This seemed to be due only partially to the slight yellowish hue imparted by the rutin. It was also evident that the vessels of the ears appeared more constricted after the rutin injection. These effects were particularly noticeable for the first hour, after which time the rutin treated animals show slowly increasing discoloration. Minor skin abrasions or injuries show up particularly well the reduction of blueness in the rutin treated animals as compared with untreated controls.

These experiments were further elaborated by using intradermal injections of sodium bisulfite. Tainter (12) had already shown that this procedure permits the demonstration of skin irritation by the trypan blue test. In our experiments we have used white albino rabbits which were injected with sodium bisulfite concentrations of 0.3 per cent to 0.6 per cent in isotonic saline solution. Intradermal wheals of 0.25 cc. each were made on the belly and the ear of such animals. This was followed 15 minutes later by 5 cc. of a 1 per cent trypan blue solution intravenously. In 8 rabbits it was found that 0.3 per cent sodium bisulfite caused marked blueness on the site of the wheal in approximately 80 per cent of the cases; a 0.6 per cent solution always caused blueness. Other rabbits were treated identically, but 200 mgm. rutin were given intravenously 20 minutes after the intradermal injections and 15 minutes prior to the injection of the dye. This resulted in the prevention of dye appearance in most of the wheals injected with 0.3 per cent of sodium bisulfite and great reduction of the blueness in the rest. There was also prevention of dye appearance in some of the places injected with 0.6 per cent of sodium bisulfite and a definite reduction in most of the others.

The above described experiments had shown that rutin reduces the systemic toxicity of procaine with or without bisulfite, but not the toxicity mefrazol. The effect of these two drugs upon capillary permeability was studied using the abdominal skin of white guinea pigs rather than rabbits. Intradermal injections of 0.2 cc. were made with 2 per cent procaine or metrazol, each with and without 0.3 per cent sodium bisulfite. This concentration of sodium bisulfite alone produces only a faint degree of blueness after injection of trypan blue. About 5 to 10 minutes after the injection of the wheals 1 to  $1\frac{1}{2}$  cc. of a 1 per cent solution of trypan blue was injected intravenously. Five guinea pigs were used for these experiments. Metrazol, with or without sodium bisulfite, caused no or only a slight bluish discoloration at the places of the wheals in 3 of these animals. In

4 of them procaine alone caused a definite bluing of the wheals; this was more pronounced with procaine plus sodium bisulfite.

**DISCUSSION.** The terms capillary fragility and capillary permeability are rather loosely used as pointed out by Landis (13). Clinically, rutin is used for the treatment of capillary fragility and the tests which have been devised to signify the presence of an increased capillary fragility, as for instance the Gothlin (14) index, are certainly more apt to measure this quality than a permeability effect. Only recently Shanno, Griffith, and LaMotte (15) have indicated that patients having increased capillary fragility may also possess an increased capillary permeability as shown by measurement of the cutaneous lymphatic flow. After administration of rutin a reduction to normal values was frequently observed with both tests. Thomas (16) saw a favorable effect of rutin upon the albuminuria of one of two nephrotic patients and mentions the possibility that "fragility" and "permeability" of capillaries may be based upon some common factors. On the other hand Oersike and Ureen (17) were unable to decrease the proteinuria induced by bovine albumin in rats by rutin. The findings of Ambrose and DeEds (2) indicate the reduction of increased capillary permeability in the skin caused by local irritation or histamine injection. It is noteworthy that Harley and others (18) have failed to demonstrate a vasomotor effect of rutin and related compounds upon the mesoappendix preparation of Zweifach and Chambers. These authors have quoted earlier work with other flavones related to rutin which has yielded discordant results in the hands of various investigators. In some of our experiments a slight elevation of blood pressure followed the infusion of 3-4 mgm./kgm. per min. of rutin in cats, and some vasoconstrictor effects could be shown on the isolated frog legs. Crisman (19) claims that rutin augments the vasomotor effect of epinephrine on the mesoappendix preparation.

The effects of sodium bisulfite upon capillaries is probably closely related to a toxic increase in permeability and possibly involves an increase of local blood flow. Indeed, Tainter and associates (12) observed intense hyperemia after intradermal injections of weak concentrations of sodium bisulfite in the human forearm.

It should be realized that an essential difference must exist between the action of sodium bisulfite and of histamine inasmuch as no wheal type response occurs after intradermal injection of sodium bisulfite in concentrations of 0.1-0.6 per cent.

The action of sodium bisulfite to increase the toxicity of certain drugs, such as epinephrine and procaine, cannot be explained solely on the basis of local irritation due to physical chemical properties. This has been shown by earlier work (7) which failed to achieve similar effects with other compounds having a corresponding acidity, reducing ability, or a combination of both. For this reason it appears justified to describe these actions of sodium bisulfite briefly as the "bisulfite phenomenon," even if the complete mode of action cannot be fully explained at the present time. Tainter (12) stated that the addition of epinephrine to the sodium bisulfite solution prevents the visible appearance of hyperemia if such mixtures are injected into the skin of the human forearm. Thus it is obvious

that epinephrine can overcome the irritating vasodilating effect of sodium bisulfite but not certain other actions which result in a faster absorption of epinephrine. This particular effect of sodium bisulfite can be successfully overcome by rutin, and a quantitative relation exists between the amounts of rutin used and the reduction of toxicity of such epinephrine-bisulfite mixtures. As discussed above, rutin may possess a slight vasoconstrictor action of its own, but this effect would certainly be negligible if compared with the powerful action of epinephrine. Inasmuch as capillary fragility is less likely to enter into the consideration of our particular experiments one may assume that the effectiveness of rutin in reducing the toxicity of epinephrine-sodium bisulfite mixtures must be directed against the phenomenon which is generally described as increased capillary permeability, lymph flow or exchange of fluid between the interstitial spaces and the circulation. A systemic antagonistic action of rutin against epinephrine has been excluded by observing that the toxicity of epinephrine hydrochloride solution is not decreased, in fact perhaps somewhat increased, by rutin. The relatively short duration of action of rutin is well illustrated in figure 1. The fact that an S-shaped curve resulted may indicate that certain specific actions of rutin are present only at higher concentrations and are rapidly lost as the tissue level decreases.

The protection obtained with rutin against the toxic and fatal effects of procaine was shown to be two fold: both against the increased toxicity caused by the addition of sodium bisulfite, and also against procaine hydrochloride itself. A systemic antagonism of rutin against procaine has been excluded by the absence of an effect of rutin upon intravenously administered procaine.

In contrast to these findings no effect of sodium bisulfite was noted upon the toxicity of metrazol nor any significant reduction of metrazol toxicity by rutin pretreatment. In order to understand the probable mechanism of action involved in these phenomena it is necessary to define some conditions which determine whether or not the toxicity of a drug will be affected by the presence of sodium bisulfite. It was found that drugs which are potentiated by bisulfite have one feature in common, namely, a wide margin between the intravenous toxicity of such compounds and their toxicity by subcutaneous or intramuscular administration. The presence of such a margin appears to be necessary but is perhaps not a sufficient condition for the demonstration of a positive "bisulfite phenomenon". As already indicated earlier (7) the fatal dose of epinephrine given intramuscularly to rats is about 15 times larger than the intravenous dose. For procaine this relation is about 33 to 1 (12). It follows that any action which greatly speeds the absorption rate will increase the toxicities of such drugs. This is precisely what sodium bisulfite does and to which rutin shows antagonism. In agreement with published data (20) we found 75 mgm./kgm. of metrazol to be fatal to 58 per cent of rats by intramuscular injection, while the intravenous lethal dose is stated to be 50 mgm./kgm. Obviously the gradient between these two doses is very small and no significant difference can be expected by an increased rate of absorption.

This still leaves unexplained the antagonistic effect of rutin against the toxic

effects of plain procaine solutions. It is well known that procedures which retard the absorption of local anesthetics decrease their systemic toxicity. The possibility of rutin exerting a slight vasoconstrictor effect by itself or sensitizing the capillaries to the constrictor action of epinephrine has been mentioned above.

Procaine in concentrations of 1 and 2 per cent exerts a definite dilating effect upon the vessels of the perfused hind legs of frogs. This effect is reduced during perfusion with a 0.05 per cent rutin solution. The large doses of procaine administered to our rats made it necessary to use 10 per cent procaine solutions. Such concentrations of procaine produce a local vasodilating effect. Additional evidence for a capillary action of procaine is to be found in the effects observed after the injection of procaine with and without sodium bisulfite in the skin of guinea pigs. In most instances a definitely increased capillary permeability is indicated by the appearance of the trypan blue at the place of injection. This was less noticeable with the use of corresponding metrazol solutions. The possibility of making use of these actions of rutin in the study of absorption of other drugs remains open for further investigation, while on the other hand the "bisulfite phenomenon" could provide a tool in the pharmacologic study of drugs which may possess rutin-like effects upon the capillaries.

#### SUMMARY

1. Reference is made to earlier work demonstrating the ability of sodium bisulfite to augment the toxicity of certain drugs by hastening their absorption from intramuscular and subcutaneous sites.

2. The increase of epinephrine toxicity in rats caused by sodium bisulfite was abolished by previous administration of rutin, whereas the toxicity of epinephrine HCl solution alone was not affected by rutin.

3. Rutin pretreatment decreased the toxicity of procaine HCl solutions with and without sodium bisulfite; in contrast, metrazol toxicity was not affected.

4. The conditions which enable sodium bisulfite to increase the toxicity of certain drugs are considered and the name "bisulfite phenomenon" is suggested for this action.

5. The effect of rutin upon certain functions of the capillaries is discussed with particular reference to the "bisulfite phenomenon".

6. The antagonism between the bisulfite phenomenon and rutin as described in this work makes feasible quantitative studies of certain actions of this flavone.

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# HINDLIMB REFLEXES OF CHRONIC SPINAL DOGS DURING CYCLES OF ADDICTION TO MORPHINE AND METHADON<sup>1</sup>

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The literature on the actions of single doses of morphine on the spinal cord has been summarized recently by Eddy (1) and Wikler (2, 3). Although addiction studies in chronic spinal animals might be expected to contribute to our knowledge of the neurophysiologic aspects of tolerance to and physical dependence upon opiate drugs, no such work has been reported before. While our investigations on morphine were in progress, we became interested in the addiction liability of methadon (6-dimethylamino-4-4-diphenyl-heptanone-3), also known as amidone, 10820, or dolophine, which was introduced in this country by Scott and Chen (4). The present paper includes the results of addiction studies on morphine and methadon in chronic spinal dogs.

**METHODS.** Twelve dogs were prepared by aseptic transection of the spinal cord between D-10 and D-12 segments under nembutal anesthesia. In the first few animals, a simple transection was made and after hemostasis was secured the muscles, fascia and skin were closed with silk sutures. In others, a segment of spinal cord 3-5 mm. in length was removed and the dura was closed with a silver clip at the site of cordectomy before closing the operative wound. No appreciable differences between the two types of preparations were noted post-operatively, but removal of a segment of cord afforded objective evidence of complete transection before autopsy.

The preparations required a good deal of care during the early post-operative period. The bladder was evacuated by manual compression several times a day, and the perineum was kept clean and dry by bathing, drying and dusting with talcum powder. Skin ulcers were treated by painting with a 2 per cent aqueous solution of gentian violet and dusting with powdered Irish moss. The animals were kept in large cages with a removable, solid galvanized iron floor which was covered with old blankets. These were changed frequently to keep the hindquarters dry. After 3-6 weeks most preparations required little care other than frequent changes of blankets. The bladder became fully automatic, the skin remained free from erosions or ulcers and the hindlimb reflexes maintained a stable level. Some of the animals learned to "walk" about the cage by raising the hindquarters with the spinal muscles above the transection until the footpads came in contact with the floor, when the positive supporting reactions came into play. Although there was no coordination between fore- and hindlimbs, the animal was able to effect progression movements of the body as a whole. Autopsies subsequently showed that the cord had been severed completely and that there was no regeneration. Several preparations are still in use 2½ years after operation. The longest surviving animal thus far lived for 3½ years after transection.

All preparations exhibited the knee jerk, ipsilateral flexor and crossed extensor reflexes, ipsilateral and crossed extensor thrusts, Philipson's reflex, scratch reflex, and also stepping movements when the lower limbs were pendent. In the lateral recumbent position, no stepping movements or other spontaneous activity occurred. In the present paper, only

<sup>1</sup> Preliminary reports: Wikler, A.: Fed. Proc., 4: 141, 1945; Wikler, A., and Frank, K.: Fed. Proc., 6: 384, 1947.

the knee jerks, ipsilateral extensor thrusts, ipsilateral flexor and crossed extensor reflexes and the spontaneous activity of the hindlimbs in the lateral recumbent position are discussed. Skin ulcers affected the reflexes markedly. If any ulcers developed during addiction studies, the dose of drug was maintained and no records of the reflexes were made until the skin had healed completely.

The hindlimb reflexes were elicited in several ways. For knee jerks, automatic devices included a solenoid hammer and a "reflex hammer" dropped a fixed distance and at a constant rate by a motor driven apparatus. The results thus obtained were not superior to tapping the patellar tendon with a "reflex hammer" by a trained technician, which was the method ultimately adopted. In the earlier experiments, the ipsilateral flexor and crossed extensor reflexes were elicited by delivering graduated faradic break-shocks from a "Harvard inductorium" to the skin of the dorsum of the ipsilateral or contralateral hindfoot through electrodes sealed to the skin with collodion and which made contact through a saline paste. Later this technique was replaced by the application of a graduated clamp for 3 seconds to the 4th toe of the ipsilateral or contralateral hindlimb. In some preparations the crossed extensor reflex was elicited by manual compression of the contralateral 4th toe. The results obtained with electrical stimulation were not superior to those obtained with clamping or manual compression, and the latter procedures were adopted as standard technique. The ipsilateral extensor thrusts were elicited by manual spreading of the toes or backward displacement of the foot pad. In all cases, the stimuli were maximal.

In recording the reflexes, no attempt was made to limit the response to one joint but the main component of each response was selected for measurement. Both "isometric" and "isotonic" methods were employed. In both cases, sufficient reproducibility of the position of the preparations in repeated experiments was obtained by using the iliac crest and sacral spine as reference points. "Isometric" recording devices included a strong spring lever and a pneumatic sylphon arrangement, the test limb being fixed rigidly to the device by a double cuff applied to the leg and foot on either side of the ankle. However, it was found that "isotonic" recording was entirely satisfactory from the standpoint of demonstrating the essential changes that occur during addiction. This method was simpler and more free from artifacts than the "isometric" devices, and was therefore utilized alone in subsequent experiments. The figures in this paper were obtained with the "isotonic" apparatus which is described in detail in figure 1.

Ordinarily, after the animal has been placed properly in position, the dowel "D" will be vertical. To allow correction for possible deviation from this position due to changes in flexor or extensor muscle tone, the maximum range of passive flexion and extension of the recording limb in each animal board position is recorded. This was not found necessary in most instances but should be done as a precaution against unanticipated changes. The recorded excursions of the limb are reduced from the actual excursion by a factor determined by the ratio C-CY:C-U, which in our experiments was 0.63. The data in the tables and illustrations refer to the recorded excursions.

In each experiment about 50 knee jerks, 10 extensor thrusts, 3-6 ipsilateral flexor, and 3-6 crossed extensor reflexes were recorded. The records were obtained with smoked paper as illustrated in figure 2. For better photographic reproducibility, India ink tracings were made (figures 3-13). These are slightly idealized in that small artifacts due to play in the roller-bearings "RB" were eliminated in the reproduction.

The effects of single doses of drugs were studied by recording spontaneous activity and reflexes of the hindlimb before and at various intervals after administration of the drug. In the case of morphine and methadon, injections were given subcutaneously and, ordinarily, the effects were recorded one hour after injection, since preliminary experiments showed that the peak effects were reached by this time. Several control observations were obtained over a period of at least one month before addiction studies were begun in any preparation.

A total of 15 complete cycles of morphine addiction were studied in 9 chronic spinal dogs. One of these preparations was subjected to 4, and another to 3 complete cycles. The usual

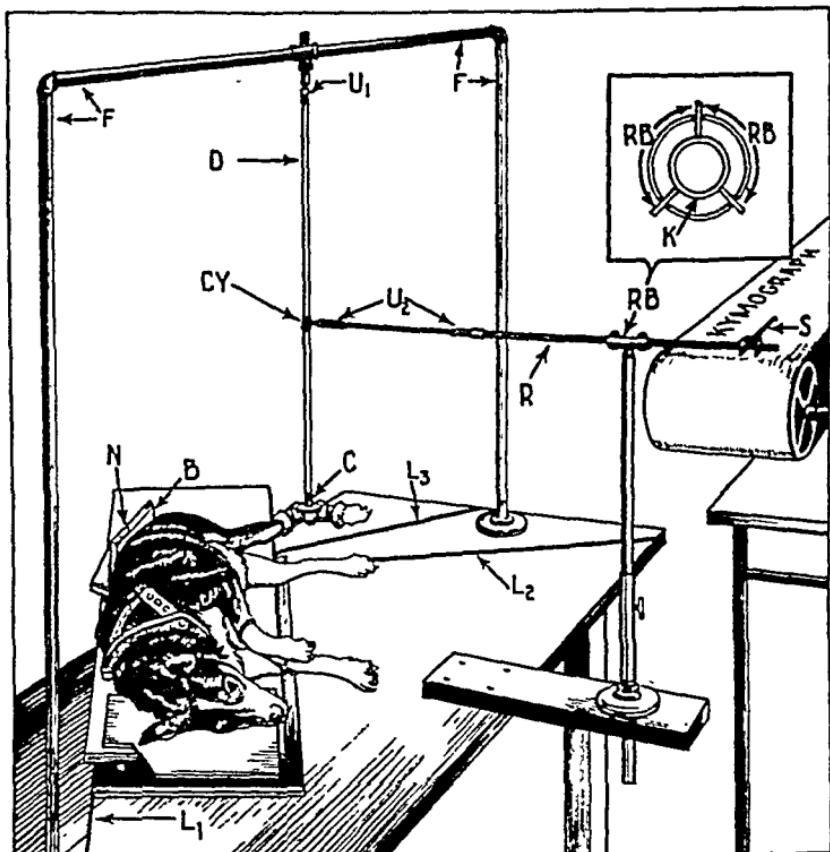


FIG. 1. DIAGRAM OF "ISOTONIC" APPARATUS FOR RECORDING HINDLIMB REFLEXES OF CHRONIC SPINAL DOGS

The preparation is placed on its right side on a portable animal board in a position such that the sacral spine is in contact with a fixed board "B", the iliac crest being in line with the notch "N". Most dogs can be trained to lie quietly in this position for hours at a time. The left lower ankle is then placed in the cuff "C", which is well padded with cotton, care being taken to keep pressure on the limb at a minimum consistent with preventing slipping within the cuff. The limb is suspended in a horizontal plane by the vertical dowel "D" from an overhead rigid frame "F". The animal board is now placed in such a position that the back border of its base is in line with one of the lines "L" ruled on the table. These lines were previously determined by placing the animal board in a position such that the main excursion of the limb during elicitation of any given reflex was in line with the long axis of the rod "R". Thus in position  $L_1$  are recorded the knee jerks and crossed extensor reflexes; in  $L_2$ , the ipsilateral extensor thrust; and in  $L_3$ , the ipsilateral flexor reflex and the spontaneous activity of the hindlimb. The movements of the rod "R" over snugly fitting roller bearings "RB" are recorded on a moving smoked paper kymograph, by the stylus "S". Universal joints at  $U_1$  and  $U_2$ , and the cylindrical sleeve bearing at "CY" eliminate strains on the rod "R" due to components of movements in directions other than that of its long axis. Although the apparatus is adjustable for dogs of different sizes, it was found more practicable to select dogs whose hindlimbs were of approximately the same length.

A cross-section through one of the two sets of roller-bearings in unit "RB" is shown in the upper right hand corner of the diagram. The two sets are about 6 cm. apart, mounted in the same rigid cylindrical frame.

procedure was to start with subcutaneous injection of morphine, 5-10 mgm./kgm. twice daily, and gradually increase the dose and frequency of injection to 25.0 mgm./kgm. every 6 hours. A few cycles of addiction were studied with one dose daily (final dose 100 mgm./kgm.) and two doses daily (final dose 50 mgm./kgm.). The addiction periods varied in length from 29-103 days each. During the addiction period, records were taken once or twice a week before and after a regular dose of morphine. When it was evident that partial tolerance to the effects on some of the reflexes had developed to the desired dose, injections of morphine were terminated abruptly and records of the reflexes and spontaneous activity of the hindlimbs were taken at intervals of about 6 hours (sometimes more often) until the

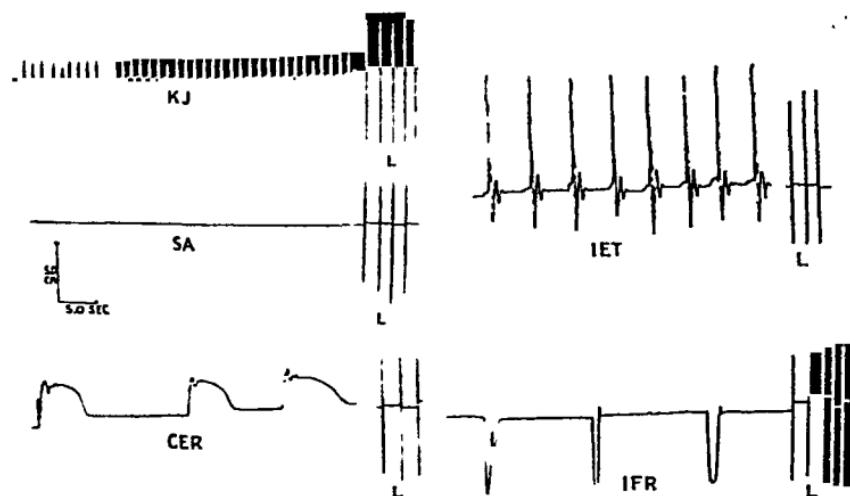


FIG. 2. DOG NO. 88 (CHRONIC SPINAL). HINDLIMB REFLEXES 14 MONTHS AFTER TRANSECTION OF SPINAL CORD AT D-10 LEVEL

KJ, knee jerks; SA, spontaneous activity; CER, crossed extensor reflex; IET, ipsilateral extensor thrust; IFR, ipsilateral flexor reflex; L, limits of passive extension (upwards) and flexor (downwards) in each recording position. Note absence of spontaneous activity. Note also that IET responses apparently exceed limits of passive extension of limb. This is due to rotation of spine during IET.

abstinence changes abated. After this, records were taken once or twice daily for the first 2 weeks, and then, once or twice weekly until a stable level was attained.

Six complete cycles of methadon addiction were studied in 5 chronic spinal dogs. The initial dose was 1.0 or 2.0 mgm./kgm. injected subcutaneously every 6 hours for periods varying from 47-63 days. The final dose level was 2.0 mgm./kgm. every 6 hours in 4 of the addiction studies, and 5.0 mgm./kgm. every 6 hours in 2 of the addiction studies. After partial tolerance to these doses had developed, methadon injections were discontinued abruptly. Records were taken during addiction and after cessation of injections as described above for morphine.

**RESULTS.** Predictability of reflexes. In table 1 are presented data on the reproducibility of the reflexes studied with the "isotonic" apparatus. The duration of the knee jerks and ipsilateral extensor thrusts are not subjected to analysis since they were very short and, ordinarily, little affected by morphine or methadon. The duration of the crossed extensor reflex, however, tended to increase with each successive elicitation of the response in any one experiment, as

indicated by the half-times of the 1st, 2nd and 3rd responses in table 1. This was also seen at times, but not consistently, in the case of the ipsilateral flexor

TABLE 1

Dog #88 (Chronic Spinal). Reproducibility of hindlimb reflexes (control measurements)

REFLEX			KNEE JERK	IPSILATERAL EXTENSOR THRUST	IPSILATERAL FLEXOR REFLEX			CROSSED EXTENSOR REFLEX		
					1st	2nd	3rd	1st	2nd	3rd
Average number of reflexes elicited per experiment			49.3	16.7						
One experiment	Amplitude	Mean (cm.)	2.25	8.03	7.92	7.42	7.64	4.49	5.48	5.45
		Mean per cent deviation	7.2	16.3	x	x	x	x	x	x
Two experiments in one day	Half-time	Mean (sec.)	x	x	1.50	1.30	0.83	3.01	4.64	5.24
		Mean per cent deviation	x	x	x	x	x	x	x	x
Twenty-one experiments in one year	Amplitude	Mean (cm.)	2.28	7.92	7.95	8.25	7.98	4.55	5.20	5.62
		Mean per cent deviation	10.0	13.7	2.5	0.0	6.0	6.2	6.7	3.2
	Half-time	Mean (sec.)	x	x	0.63	0.55	0.43	2.08	4.63	5.75
		Mean per cent deviation	x	x	20.6	27.2	7.0	31.4	12.6	2.8

"Amplitude" refers to the maximal excursion of the hindlimb on eliciting the reflex indicated and using the "isotonic" recording apparatus described in the text. "Half-time" refers to the time required for the response to return to half of its maximal value. The mean values for "One Experiment" and "Twenty-one Experiments in one year" are the same, i.e., the average of the means for each of the 21 experiments. The mean per cent deviations for "One Experiment" represent the average of the mean per cent deviations in each of the 21 experiments, the deviations being referred to the mean for each particular experiment. In calculating the mean per cent deviations for "Twenty-one Experiments in one year" the mean for all the 21 experiments is utilized and the average deviation of each measurement in all 21 experiments is obtained with reference to this mean. In "Two Experiments in one day", the mean values represent the average of the means for 4 experiments performed in groups of 2 in 2 days. The mean per cent deviations in this category are obtained by averaging the mean per cent deviations for each of the 2 days. These deviations are obtained by averaging the deviations of each measurement from the mean for that day.

reflex. This facilitatory effect on the after-discharge of the reflex could be abolished by flexing the responding limb passively. Examination of table 1 shows that the reflexes are predictable within limits which vary with each reflex

and the span of time under consideration—i.e., 1 experiment, 2 experiments in 1 day, or numerous experiments over as long as 1 year. Most addiction studies covered a total period of 3 or 4 months. The drug effects which are regarded as significant in this paper exceed spontaneous variations by a wide margin. Another criterion of significance is the return to its control value, after drug effects presumably have worn off, of any reflex or spontaneous activity exhibiting a change attributed to the effects of the drug.

**EFFECTS OF SINGLE DOSES OF MORPHINE AND METHADON.** As shown in table 2, the effects of morphine and methadon were essentially the same, namely, consistent depression or abolition of the flexor and crossed extensor reflexes and stepping movements; small but variable effects on the knee jerks; and, in most

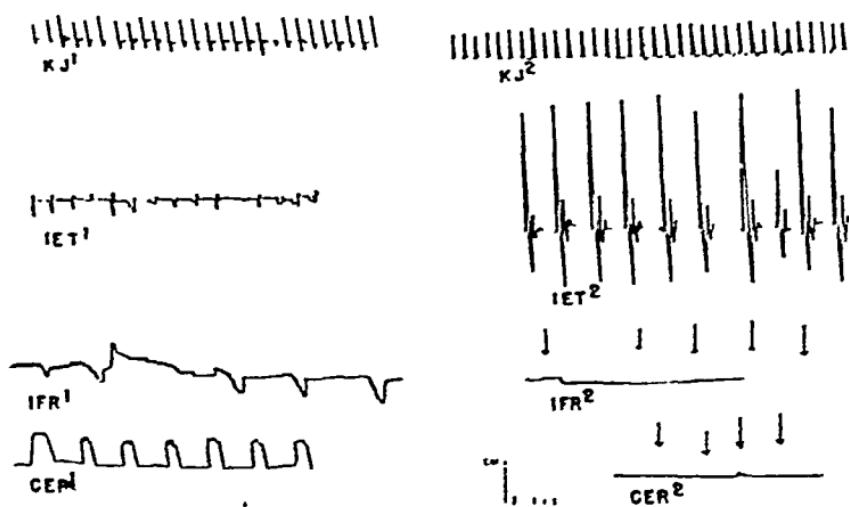


FIG. 3. Dog No. 80 (CHRONIC SPINAL). EFFECTS OF METHADON, 5.0 MG/M./KGM. ON HINDLIMB REFLEXES

Superscripts 1 and 2 refer respectively to before and one hour after subcutaneous injection of the drug. The knee jerks (KJ) are essentially unchanged. The ipsilateral extensor thrusts (IET) could not be elicited before injection but are very active after methadon. The ipsilateral flexor (IFR) and crossed extensor (CER) reflexes are abolished by the drug. (Arrows indicate stimuli when responses are absent).

instances, enhancement of the ipsilateral extensor thrust. The last effect could be demonstrated best in preparations in which the ipsilateral extensor thrust was relatively weak (as was frequently the case in the early postoperative period). After morphine or methadon, the ipsilateral extensor thrust response was enhanced many fold (figure 3). In preparations with maximal extensor thrusts, the enhancement produced by morphine or methadon was manifested by a decrease in threshold—i.e., the response could be elicited by merely touching (displacement?) the hairs on the sole of the foot. In a few instances, morphine or methadon depressed the ipsilateral extensor thrust. Another curious atypical effect was noted occasionally, namely, an alteration in pattern of the response. After morphine or methadon, extension was replaced by flexion, particularly at

the hip. At the same time, the identical response (ipsilateral flexor reflex) to the clamping of a toe was abolished. The effects on the knee jerk were more variable; in most cases it appeared to be unaffected. However, when as in some preparations, the knee jerk exhibited a relatively long duration (after-discharge?), the latter was markedly shortened by morphine or methadon regardless of the effects on amplitude.

Increase in the size of the dose of morphine or methadon prolonged the effects (e.g., depression of the ipsilateral flexor reflex for 2 days after morphine, 50 mgm./kgm.), but these did not differ qualitatively from the effects of smaller doses. In a few records, after recovery from the larger doses, the flexor reflex seemed to be somewhat larger than the control values.

With very large doses of morphine (100-150 mgm./kgm.) or methadon (40-50 mgm./kgm.), convulsions appeared  $\frac{1}{2}$  to  $1\frac{1}{2}$  hours after subcutaneous injection.

TABLE 2

*Effects of single doses of morphine and methadon on hindlimb reflexes on non-tolerant chronic spinal dogs*

REFLEX	MORPHINE (12 DOGS; 60 EXPERIMENTS) PERCENT EXPERIMENTS			METHADON (9 DOGS; 18 EXPERIMENTS) PERCENT EXPERIMENTS		
	enhanced	unchanged	depressed	enhanced	unchanged	depressed
Knee jerks.....	36.6	45.0	18.4	27.8	55.5	16.7
Ipsilateral extensor thrust.....	87.4	10.8	1.8	77.7	5.6	16.7
Ipsilateral flexor reflex.....	0.0	0.0	100.0	0.0	0.0	100.0
Crossed extensor reflex.....	0.0	0.0	100.0	0.0	0.0	100.0
Stepping movements*.....	0.0	0.0	100.0	0.0	0.0	100.0

\* Present only when hindlimbs are unsupported and in dependent position.

Notes: When the knee jerk response was sustained, morphine or methadon converted it into a simple twitch. On attempting to elicit the ipsilateral extensor thrust in 4 instances after morphine and 2 after methadon, a strong hip-flexion component appeared, even though the same response to pinching the toe (ipsilateral flexor reflex) was depressed or abolished.

These convulsions were tonic and clonic and were confined to the segments of the body *rostral* to the level of spinal transection. In the hindlimbs, only the changes noted after smaller doses were seen. At no time did any dose of morphine produce spontaneous activity of the hindlimbs.

Morphine or methadon also lowered body temperature and pulse rate, and had variable effects on respiration. These changes will be described in greater detail in another paper (5).

EFFECTS OF REPEATED DOSES OF MORPHINE AND METHADON. The changes in reflexes and spontaneous activity of the hindlimbs, and the effects of morphine thereon during addiction are shown in figures 4-8, A-C. No spontaneous activity of the hindlimbs appeared while the preparations were receiving morphine. Partial tolerance to the depressant effects of any given dose of the drug on the flexor and crossed extensor reflexes developed within 2-3 weeks. When the dose was increased, reflex depression increased, but eventually partial toler-

ance developed to the new dose. As addiction progressed, the flexor and crossed extensor reflexes became hyperactive before each regular dose of morphine. This was manifested by increase in amplitude and duration of the responses as well as obviously decreased thresholds. In contrast, knee jerks and ipsilateral extensor thrusts showed little change during this period. Tolerance to the stimulant effects of morphine on the extensor thrusts did not develop. A statement regarding tolerance to the effects of morphine on the knee jerk cannot be made because of their variability and the small effects of the drug on this reflex.

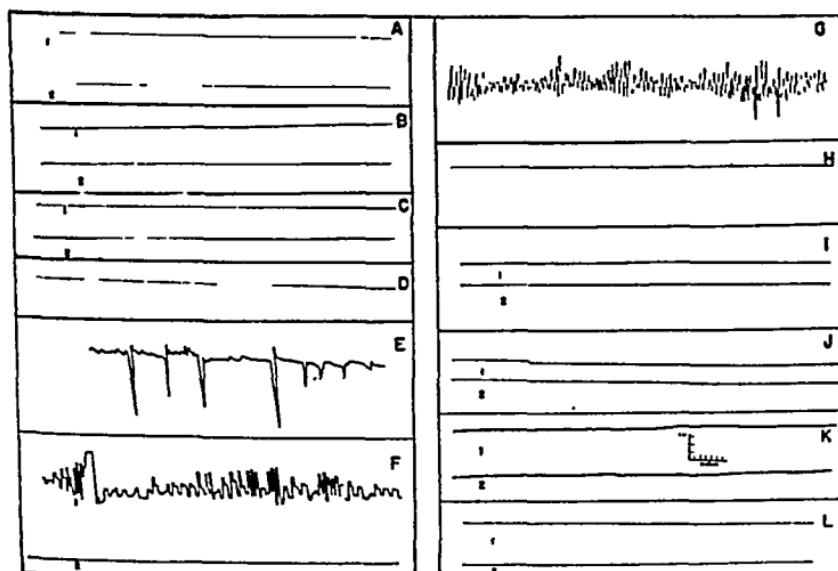


FIG. 4. DOG NO. 88 (CHRONIC SPINAL). SPONTANEOUS ACTIVITY OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts 1 and 2 refer respectively to before and 1 hour after injection. A-D, no spontaneous activity. A, pre-addiction, effects of morphine, 5.0 mgm./kgm.; B, 16th day of addiction, dose level 10.0 mgm./kgm. every 6 hours; C, 53rd day of addiction, dose level 25.0 mgm./kgm. every 6 hours; D, 24 hours abstinent; E, 30 $\frac{1}{2}$  hours abstinent. There is irregular spontaneous activity; F, 41 $\frac{1}{2}$  hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection there is continuous spontaneous rhythmic activity which ceases after injection; G, 72 hours abstinent. Spontaneous activity has increased in amplitude and is more regular; H-L, no spontaneous activity; H, 41 $\frac{1}{2}$  days abstinent; I, 7 $\frac{1}{2}$  days abstinent; J, 0 $\frac{1}{2}$  days abstinent; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm.; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm.

Tolerance also developed to the temperature-lowering effects of morphine but not to its effects on the pulse rate.

The development of tolerance to the effects of methadon on the hindlimb reflexes was slower but similar in all other respects to that of morphine (figures 9, 10).

**WITHDRAWAL OF MORPHINE AND METHADON.** The changes in the hindlimbs on withdrawal of morphine or methadon were similar in every respect except time course, and will be described together (figures 4-8, D-L, and figure 11). The earliest change was a marked exaggeration of the flexor and crossed extensor

reflexes which were increased in amplitude and duration and also became repetitive, the response to each stimulus being a rapid series of flexions and extensions followed by prolonged flexion (flexor reflex) or extension (crossed extensor reflex). In some preparations this pattern was "normal" in the control series, but was intensified during withdrawal. Concomitantly, the knee jerks and ipsilateral extensor were markedly reduced. These changes progressed with time and as early as 30½ hours after morphine withdrawal and 9 hours after methadon with-

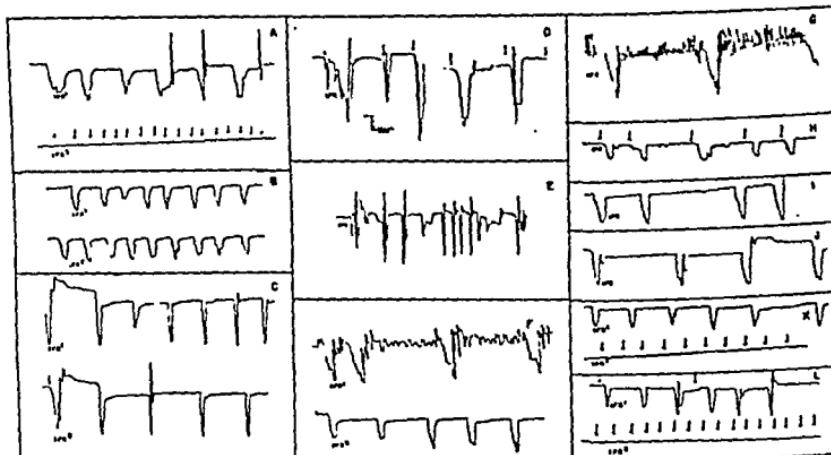


FIG. 5. DOG NO. 88 (CHRONIC SPINAL). IPSILATERAL FLEXOR REFLEX (IFR) OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts 1 and 2 refer respectively to before and 1 hour after subcutaneous injection. Arrows indicate stimuli when responses are absent, unclear or repetitive. A, pre-addiction. Effects of morphine, 5.0 mgm./kgm. IFR is abolished; B, 16th day of addiction, dose level 10.0 mgm./kgm. every 6 hours. Marked tolerance has developed to the depressant action of morphine on IFR; C, 53rd day of addiction, dose level 25 mgm./kgm. every 6 hours. The amplitude of IFR has increased although the duration of the response is shorter. There is marked tolerance to this large dose of morphine; D, 24 hours abstinent. IFR is exaggerated and repetitive; E, 30½ hours abstinent. There is irregular spontaneous activity. IFR is exaggerated and repetitive; F, 41½ hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection there is continuous spontaneous rhythmic activity. IFR is exaggerated, repetitive and temporarily interrupts the spontaneous activity. After injection, spontaneous activity disappears but IFR is only slightly depressed; G, 72 hours abstinent. Spontaneous activity has increased in amplitude and is more regular. IFR as in F; H, 4½ days abstinent. Spontaneous activity has ceased. IFR is smaller in amplitude; I, 7½ days abstinent. IFR is comparable to that in A; J, 9½ days abstinent. IFR same; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm. IFR is abolished. Tolerance has already been lost to a marked extent; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm. IFR is abolished, as in A.

drawal, rhythmic alternating flexion and extension of the hindlimbs appeared, at first, after elicitation of the flexor or crossed extensor responses, later, spontaneously. These spontaneous movements of the hindlimbs, with the dog in the lateral recumbent position, were similar to the "stepping movements" exhibited by "normal" chronic spinal dogs when held aloft with the hindlimbs freely pendent. At this time, little other evidence of abstinence was shown by the dogs.

By 42 hours after withdrawal of morphine or 15 hours after withdrawal of methadon these spontaneous stepping movements became so large in amplitude

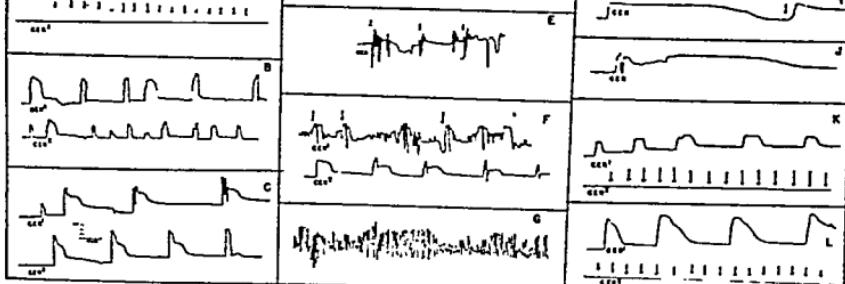


FIG. 6. Dog No. 88 (CHRONIC SPINAL). CROSSED EXTENSOR REFLEX (CER) OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts 1 and 2 refer respectively to before and 1 hour after subcutaneous injection. Arrows indicate stimuli when responses are absent, unclear or repetitive. A, pre-addiction. Effects of morphine, 5.0 mgm./kgm. CER is abolished; B, 16th day of addiction, dose level 10.0 mgm./kgm. every 6 hours. Marked tolerance has developed to the depressant action of morphine on CER; C, 53rd day of addiction, dose level 25.0 mgm./kgm. every 6 hours. There is marked tolerance to this large dose of morphine; D, 24 hours abstinent. The duration of the response is greatly prolonged; E, 30½ hours abstinent. There is irregular spontaneous activity. CER is repetitive; F, 41½ hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection there is continuous spontaneous rhythmic activity. CER is repetitive and temporarily interrupts spontaneous activity. After injection, spontaneous activity ceases but CER is only slight depressed; G, 72 hours abstinent. Spontaneous activity has increased in amplitude and is more regular. CER is obscured; H, 4½ days abstinent. Spontaneous activity has ceased. CER is smaller in amplitude; I, 7½ days abstinent. The duration of CER is markedly prolonged; J, 9½ days abstinent. CER is still of long duration; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm. CER is abolished. Tolerance has already been lost to a marked extent; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm. CER is abolished. Note that in K and L, pre-injection KJ is comparable to that in A.

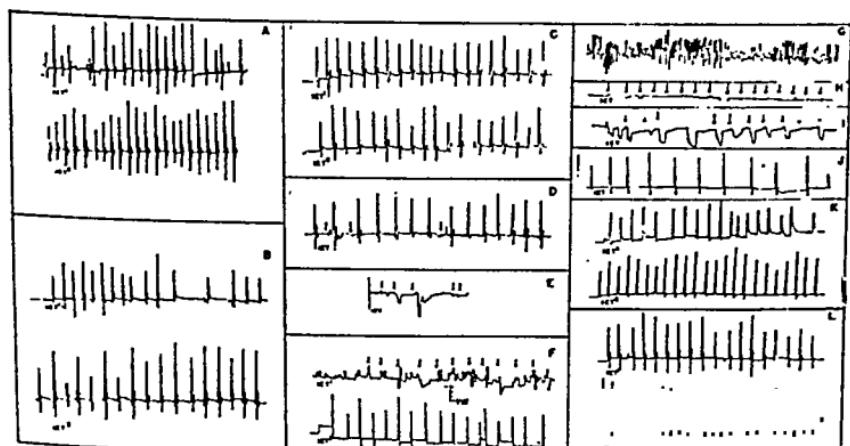


FIG. 7. Dog No. 88 (CHRONIC SPINAL). IPSILATERAL EXTENSOR THRUST (IET) OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts 1 and 2 refer respectively to before and 1 hour after subcutaneous injection. Arrows indicate stimuli when responses are absent or unclear. A, pre-addiction. Effects of morphine, 5.0 mgm./kgm. IET slightly enhanced; B, 16th day of addiction, dose level 10.0 mgm./kgm. every 6 hours. No essential change; C, 53rd day of addiction, dose level 25.0 mgm./kgm. every 6 hours. No essential change; D, 24 hours abstinent. No essential change; E, 30½ hours abstinent. There is irregular spontaneous activity. IET can not be elicited; F, 41½ hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection there is continuous spontaneous rhythmic activity. This obscures IET which, if present at all, is greatly reduced. After injection, spontaneous activity ceases and IET is restored; G, 72 hours abstinent. Spontaneous rhythmic activity has increased in amplitude and is more regular. IET can not be tested; H, 4½ days abstinent. Spontaneous activity has ceased. IET is still markedly reduced; I, 7½ days abstinent. Attempts to elicit IET evoke only flexion; J, 9½ days abstinent. IET is restored; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm. IET is slightly enhanced; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm. IET is slightly enhanced.

reflexes which were increased in amplitude and duration and also became repetitive, the response to each stimulus being a rapid series of flexions and extensions followed by prolonged flexion (flexor reflex) or extension (crossed extensor reflex). In some preparations this pattern was "normal" in the control series, but was intensified during withdrawal. Concomitantly, the knee jerks and ipsilateral extensor were markedly reduced. These changes progressed with time and as early as 30½ hours after morphine withdrawal and 9 hours after methadon with-

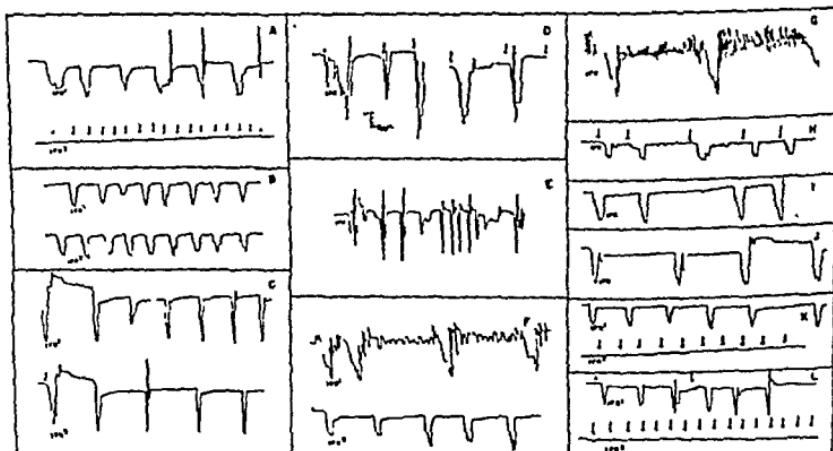


FIG. 5. DOG NO. 88 (CHRONIC SPINAL). IPSILATERAL FLEXOR REFLEX (IFR) OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts I and 2 refer respectively to before and 1 hour after subcutaneous injection. Arrows indicate stimuli when responses are absent, unclear or repetitive. A, pre-addiction. Effects of morphine, 5.0 mgm./kgm. IFR is abolished; B, 16th day of addiction, dose level 10.0 mgm./kgm., every 6 hours. Marked tolerance has developed to the depressant action of morphine on IFR; C, 53rd day of addiction, dose level 25 mgm./kgm., every 6 hours. The amplitude of IFR has increased although the duration of the response is shorter. There is marked tolerance to this large dose of morphine; D, 24 hours abstinent. IFR is exaggerated and repetitive; E, 30½ hours abstinent. There is irregular spontaneous activity. IFR is exaggerated and repetitive; F, 41½ hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection there is continuous spontaneous rhythmic activity. IFR is exaggerated, repetitive and temporarily interrupts the spontaneous activity. After injection, spontaneous activity disappears but IFR is only slightly depressed; G, 72 hours abstinent. Spontaneous activity has increased in amplitude and is more regular. IFR as in F; H, 4½ days abstinent. Spontaneous activity has ceased. IFR is smaller in amplitude; I, 7½ days abstinent. IFR is comparable to that in A; J, 9½ days abstinent. IFR same; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm. IFR is abolished. Tolerance has already been lost to a marked extent; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm. IFR is abolished, as in A.

drawal, rhythmic alternating flexion and extension of the hindlimbs appeared, at first, after elicitation of the flexor or crossed extensor responses, later, spontaneously. These spontaneous movements of the hindlimbs, with the dog in the lateral recumbent position, were similar to the "stepping movements" exhibited by "normal" chronic spinal dogs when held aloft with the hindlimbs freely pendent. At this time, little other evidence of abstinence was shown by the dogs.

By 42 hours after withdrawal of morphine or 15 hours after withdrawal of methadon these spontaneous stepping movements became so large in amplitude

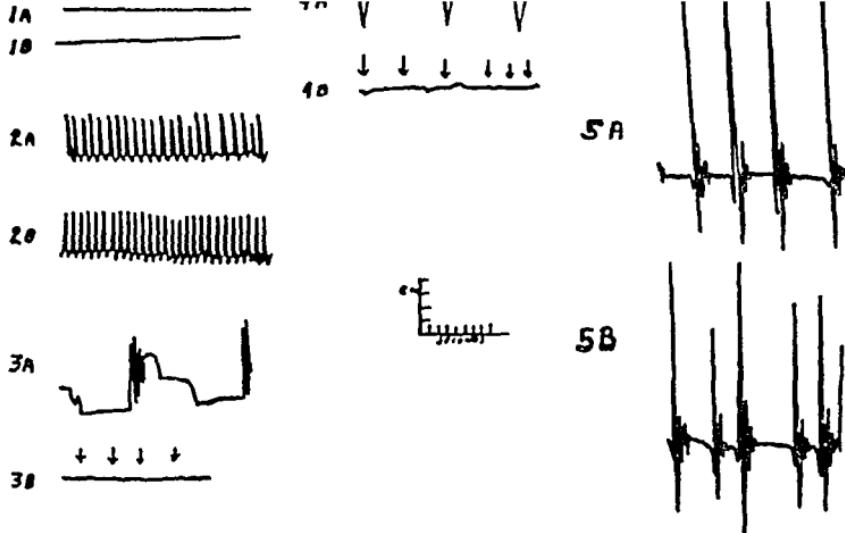


FIG. 9. Dog No. 69 (CHRONIC SPINAL). EFFECTS OF METHADON, 2.0 MG.M./KGM., ON HINDLIMB REFLEXES BEFORE ADDICTION

A and B are, respectively, responses before and 1 hour after subcutaneous injection of the drug. Arrows indicate stimuli when responses are absent. 1, spontaneous activity; 2, knee jerks; 3, crossed extensor reflex; 4, ipsilateral flexor reflex; 5, ipsilateral extensor thrust. Note depression of all reflexes except the knee jerk. (The slight depressant action on the ipsilateral extensor thrust is atypical; in most chronic spinal preparations this reflex is enhanced by methadon).

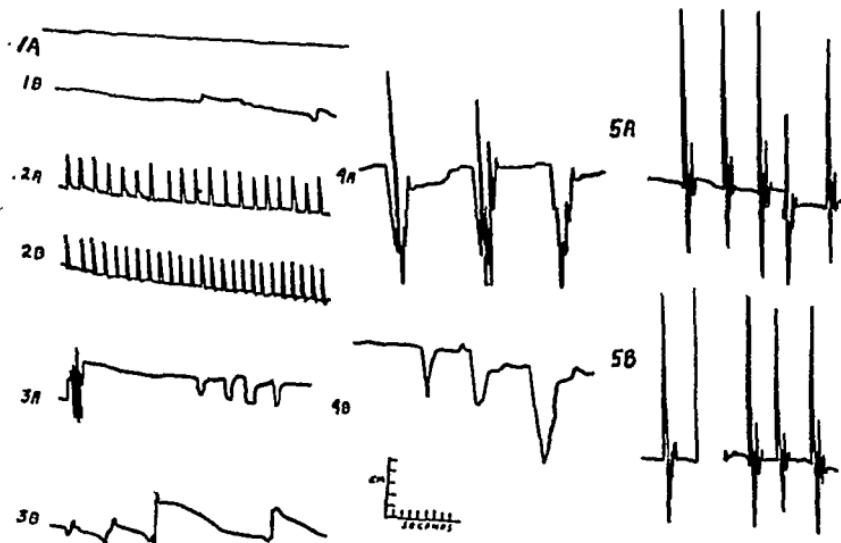


FIG. 10. Dog No. 69 (CHRONIC SPINAL). PARTIAL TOLERANCE TO DEPRESSANT EFFECTS OF METHADON ON HINDLIMB REFLEXES ON 45TH DAY OF ADDICTION (DOSE LEVEL 2.0 MG.M./KGM. EVERY 6 HOURS)

A and B are respectively responses before and 1 hour after subcutaneous injection of methadon, 2.0 mgm./kgm. 1, spontaneous activity; 2, knee jerks; 3, crossed extensor reflex; 4, ipsilateral flexor reflex; 5, ipsilateral extensor thrust. Note: Spontaneous random movements (in part transmitted from the body of the dog) due to increased restlessness are only slightly diminished by methadon. The pre-injection crossed extensor reflex is repetitive, exhibits prolonged after-discharge and is followed by rhythmic alternating flexion and extension movements. After methadon there is considerable reduction in the response but the reflex is not abolished. Likewise, the ipsilateral flexor reflex is exaggerated and repetitive and is only moderately depressed by methadon. The ipsilateral extensor thrust and the knee jerk are unaffected.

and rapid in frequency that it was impossible to test knee jerks or ipsilateral extensor thrusts. Flexor and crossed extensor reflexes were markedly exaggerated and temporarily replaced the spontaneous activity.

At this time the dogs showed other signs of abstinence, varying considerably in severity from one animal to another. These included tachycardia, elevation of rectal temperature ( $1^{\circ}$  to  $2^{\circ}\text{C}.$ ), panting, vomiting, yawning, lacrimation,

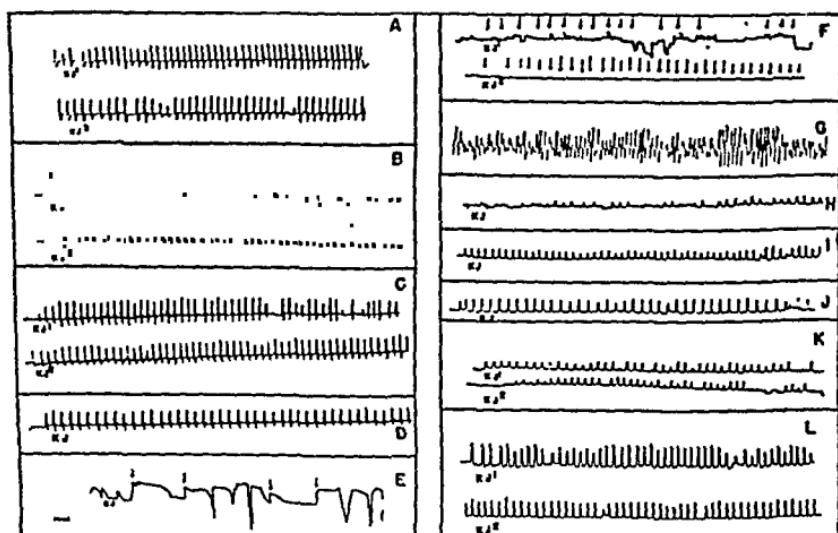


FIG. 8. DOG NO. 88 (CHRONIC SPINAL). KNEE JERK (KJ) OF HINDLIMB DURING CYCLE OF MORPHINE ADDICTION

Superscripts 1 and 2 refer respectively to before and 1 hour after subcutaneous injection. Arrows indicate stimuli when responses are absent or unclear. A, pre-addiction. Effects of morphine, 5.0 mgm./kgm. No essential change; B, 16th day of addiction, dose level 10.0 mgm./kgm. every 6 hours. No essential change after injection. The differences in amplitude of KJ between A and B are not greater than spontaneous variation over a comparable period of time; C, 53rd day of addiction, dose level 25.0 mgm./kgm. every 6 hours. No essential change; D, 24 hours abstinent. No essential change; E, 30½ hours abstinent. There is spontaneous irregular activity, making it difficult to elicit KJ; F, 41½ hours abstinent. Effects of morphine, 25.0 mgm./kgm. Before injection, there is continuous spontaneous rhythmic activity. KJ can not be elicited. After injection, spontaneous activity ceases, but KJ is still absent; G, 72 hours abstinent. Spontaneous activity has increased in amplitude and is more regular. KJ can not be elicited; H, 4½ days abstinent. Spontaneous activity has ceased. KJ can not be elicited but is of small amplitude; I, 7½ days abstinent. KJ is partially restored; J, 9½ days abstinent. KJ somewhat greater in amplitude; K, 36 days abstinent. Effects of morphine, 10.0 mgm./kgm. No essential change; L, 47 days abstinent. Effects of morphine, 5.0 mgm./kgm. No essential change after injection. Note amplitude of KJ is comparable to that in A.

rhinorrhea, and occasionally diarrhea. Another striking withdrawal phenomenon was the presence of palpable and, at times, visible muscle tremors. In the chronic spinal dogs these could be palpated at different times above or below the level of the spinal transection.

The peak of the abstinence syndrome was generally reached by the 72nd-90th hour after withdrawal of morphine, and by the 24th-30th hour after withdrawal

of methadon. Thereafter the general signs of abstinence (tachycardia, fever, vomiting, etc.) decreased rapidly while the changes in the hindlimbs disappeared more slowly. Generally, immediately after the spontaneous activity of the hindlimbs disappeared, all the hindlimb reflexes were subnormal for a day or two. After this period, the flexor and crossed extensor reflexes became supernormal, while the knee jerks and ipsilateral extensor thrusts remained reduced. The general signs of abstinence were generally over by the 5th day after withdrawal of morphine and the 3rd day after withdrawal of methadon. The hindlimbs returned to the approximate pre-addiction level 10-14 days after withdrawal of morphine or methadon, but in the case of morphine, slight changes such as en-

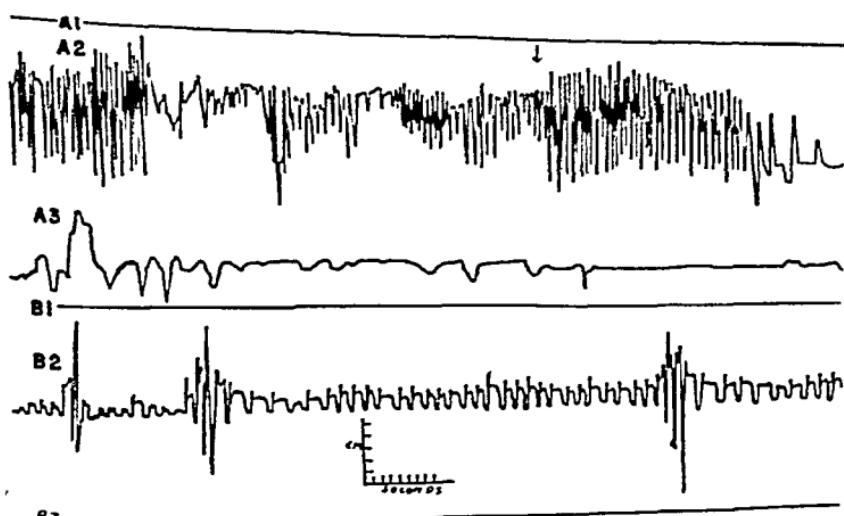


FIG. 13. SIMILARITY OF ACUTE EFFECTS OF ESERINE TO MORPHINE ABSTINENCE SYNDROME IN HINDLIMBS OF CHRONIC SPINAL DOGS, AND EFFECTS OF METHADON ON RUNNING MOVEMENTS INDUCED BY ESERINE

A-1, Dog No. 70 (chronic spinal, D-10), spontaneous activity before eserine; A-2, 20 minutes after subcutaneous injection of eserine, 0.25 mgm./kgm. At arrow, methadon, 5.0 mgm./kgm., was given intravenously; A-3, continuous from A-2. Note rhythmic running movements induced by eserine, and prompt abolition of the same by methadon; B-1, Dog No. 18 (chronic spinal, D-10), spontaneous activity before addiction; B-2, 29 hours abstinent (after 50 days addiction to morphine sulfate, final dose level 10.0 mgm./kgm. 4 times daily); B-3, 116 hours abstinent. Note spontaneous running movements of hindlimbs during early period of morphine withdrawal and spontaneous subsidence of same later.

hancement of the flexor reflexes persisted for variable times, up to 4 months following withdrawal. This apparent difference in post-withdrawal effects after morphine and methadon addiction, however, may not be significant because of the smaller number of addiction studies with methadon.

Morphine or methadon in significant amounts, abolished the abstinence signs produced by withdrawal of the other drug (figure 12). The effects of one or the other drug on the hindlimb reflex abstinence syndrome were indistinguishable from each other. Either morphine or methadon abolished the spontaneous activity of the hindlimbs as well as the general signs of abstinence, and restored the hindlimb reflexes to their control-values.

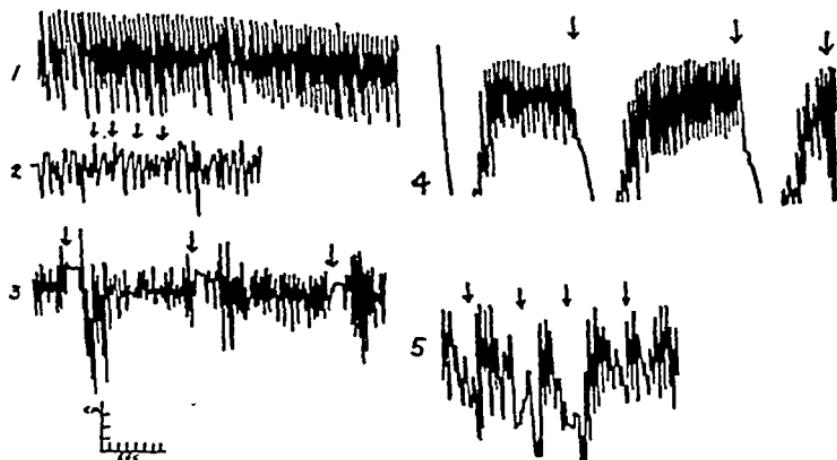


FIG. 11. DOG NO. 69 (CHRONIC SPINAL). METHADON "ABSTINENCE SYNDROME" IN HINDLIMBS 42 HOURS AFTER LAST INJECTION (45 DAY ADDICTION PERIOD); FINAL DOSE LEVEL 2.0 MGm./KGM. EVERY 6 HOURS

1, spontaneous activity; 2, knee jerk; 3, crossed extensor reflex; 4, ipsilateral flexor reflex; 5, ipsilateral extensor thrust. Arrows indicate stimuli. Note: Spontaneous rhythmic alternating flexion and extension movements are continuous and obscure any knee jerks which may be present. Stimuli which had evoked the crossed extensor reflex now inhibit the spontaneous activity temporarily. The ipsilateral flexor reflex is exaggerated (off the recording paper) and prolonged. The ipsilateral extensor thrust is absent, and stimuli which have previously evoked it now elicit ipsilateral flexion.

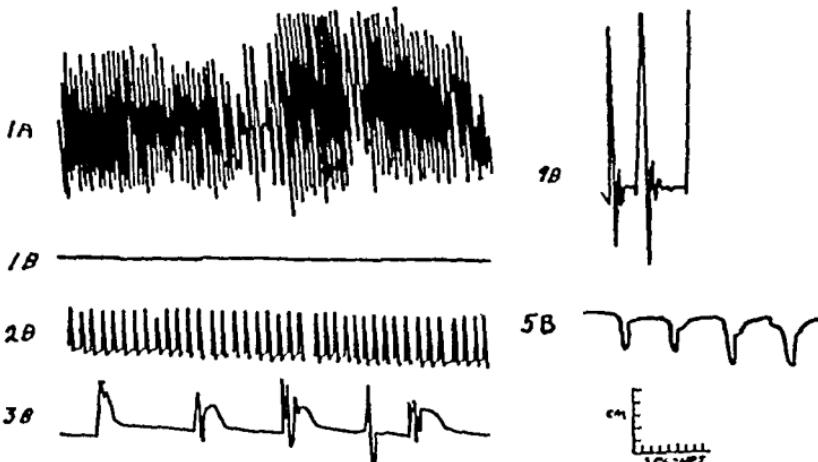


FIG. 12. DOG NO. 69 (CHRONIC SPINAL). EFFECTS OF MORPHINE ON METHADON ABSTINENCE SYNDROME IN HINDLIMBS 17 HOURS AFTER LAST INJECTION OF METHADON ON 35TH DAY OF ADDICTION (DOSE LEVEL 2.0 MGm./KGM. EVERY 6 HOURS)

A and B are, respectively, responses before and 1 hour after subcutaneous injection of morphine sulfate, 10.0 mgm./kkgm. 1, spontaneous activity; 2, knee jerk; 3, crossed extensor reflex; 4, ipsilateral extensor thrust; 5, ipsilateral flexor reflex. Note: This amount of morphine abolishes the spontaneous rhythmic running movements but not the ipsilateral flexor and crossed extensor reflexes. The latter are regularly abolished by such a dose of morphine in the non-addicted preparation.

system. Further elucidation of this problem may be achieved by examining the effects of morphine or methadon in chronic spinal dogs on action potentials evoked by elicitation of the various hindlimb reflexes.

The observations on the effects of convulsing doses of morphine or methadon in chronic spinal dogs are in agreement with those of Leimdorfer (7) on the action of methadon on the electroencephalogram and electrospinogram of curarized cats. In these species, at least, the convulsions induced by morphine or methadon are apparently of cortical, not spinal, origin.

The specificity of the action of morphine and methadon on the hindlimb reflexes of the chronic spinal dog has not been fully investigated. However, studies on one barbiturate (sodium pentobarbital) indicate that this drug has only a depressant effect on all reflexes, although the flexor and crossed extensor reflexes are abolished before the knee jerk and ipsilateral extensor thrust. This is in agreement with previous studies on the acute spinal cat with action potential techniques (3).

The demonstration of tolerance and physical dependence to morphine and methadon in the chronic spinal dog affords unequivocal evidence that the spinal cord is involved to at least some degree in addiction to these drugs, and presumptive evidence that the spinal cord is one of the portions of the nervous system which give rise to the withdrawal phenomena. It is well known that restlessness and muscular twitching are outstanding phenomena in the human morphine withdrawal syndrome. The addict's phrase, "kicking a habit," as a term for undergoing withdrawal from opiates, is derived from the muscular twitches, especially in the lower extremities, which he exhibits during this period. These may well originate in the spinal cord. However, the spinal cord below the level of transection in our dogs could still be affected indirectly by some of the withdrawal phenomena arising from changes above the level of transection, such as elevation of body temperature, humoral agents, or afferents to the spinal cord from visceral disturbances. However, artificially induced fever had no effect on the hindlimbs of chronic spinal dogs. Cross-circulation experiments and addiction studies in preparations in which visceral afferents below the level of spinal cord transection have been sectioned, are needed to rule out the other possibilities with certainty. However, the difference in the time course of the general abstinence syndrome and that of the hindlimbs makes such indirect effects improbable.

With reference to the application of our findings to the site of origin of the abstinence syndrome in man, it is also necessary to bear in mind the possibility that, in the intact organism, the direct effects of drugs on the spinal cord may be masked by inhibitory or facilitatory influences from higher segments of the neuraxis, which may or may not be affected simultaneously by the agent. Also the problem of telencephalization must be considered. In the ascending evolutionary scale, physiological functions are shifted more and more in the direction of the cerebral cortex, and this region of the central nervous system is generally most sensitive to chemical agents. Hence conclusions regarding the site of

OTHER AGENTS. The effects of sodium pentobarbital, neostigmine, eserine, and hyperthermia were studied in chronic spinal dogs which were not receiving any drugs.

*Sodium Pentobarbital.* In 6 experiments, single intravenous injections of sodium pentobarbital, 8 mgm./kgm., abolished the flexor and crossed extensor reflexes and depressed slightly the knee jerk and ipsilateral extensor thrust. After 15 mgm./kgm. these reflexes were markedly depressed, and after 30 mgm./kgm., all reflex activity in the hindlimbs disappeared except, at times, a very feeble knee jerk.

*Neostigmine.* In 3 experiments, subcutaneous injection of neostigmine 0.1-0.3 mgm./kgm., was followed by enhancement of all hindlimb reflexes and the appearance of irregular spontaneous movements of the hindlimbs.

*Eserine.* In 6 experiments, subcutaneous injection of eserine, 0.25-0.50 mgm./kgm., produced spontaneous movements of the hindlimbs with some variability in pattern. In some experiments, irregular extensor or flexor movements of both hindlimbs appeared spontaneously, while in others, the movements were rhythmic and alternating, resembling strongly the spontaneous activity of the hindlimbs during withdrawal from morphine or methadon. The spontaneous activity induced by eserine was abolished by subcutaneous injection of either morphine, 10.0 mgm./kgm., or methadon, 5.0 mgm./kgm. (figure 13). The effects of smaller doses of eserine on the hindlimb reflexes were variable, contrasting with the uniformly enhancing effects of neostigmine.

*Hyperthermia.* Rectal temperature of 2 chronic spinal dogs was raised by heating in an incubator to 40.3° and 40.7°C. respectively (normal rectal temperature 38.5° to 39.0°C.). In another chronic spinal dog, the temperature was raised to 39.1°C. by intravenous injection of typhoid vaccine. No changes were noted in the hindlimbs.

**DISCUSSION.** Some similarities and some differences may be noted on comparing the actions of single doses of morphine and methadon in the chronic spinal dog with those in the acute spinal cat (2, 3). In both, all responses to nociceptive stimulation (flexor and crossed extensor reflexes) are markedly depressed. On the other hand, whereas the knee jerk of the acute spinal cat either is not affected or is enhanced, that of the chronic spinal dog is depressed in an appreciable percentage of experiments (table 2). Depressant effects of morphine on the knee jerks of spinal dogs were reported by Luckhardt and Johnson (6). However, this occurred in only 18.4 per cent of our experiments.

The effects of morphine or methadon on the ipsilateral extensor thrust appear not to have been described before. It may be of some significance that in the chronic spinal dog the reflexes which are most markedly and consistently depressed by these drugs are characterized by a well-marked after-discharge (ipsilateral flexor and crossed extensor reflexes), whereas those reflexes which are unaffected or enhanced have little or no after discharge (ipsilateral extensor thrust and most knee jerks). This suggests that the locus of the depressant effects of morphine or methadon is primarily upon some internuncial neurone

## SUMMARY

1. Methods for preparing long-surviving, chronic spinal dogs, and methods for recording spontaneous activity and hindlimb reflexes in such preparations are described.

2. In chronic spinal dogs, single doses of morphine or methadon depress markedly the ipsilateral flexor and crossed extensor reflexes, enhance the ipsilateral extensor thrust in most instances, and have small but variable effects on the knee jerk. After very large doses of morphine (100-150 mgm./kgm.) or methadon (40-50 mgm./kgm.) tonic and clonic convulsions appear in the segments rostral to the transection but not below it.

3. During addiction to morphine or methadon, tolerance develops to the depressant effects of morphine and methadon on the ipsilateral flexor and crossed extensor reflexes, but not to the excitant effects on the ipsilateral extensor thrust. As addiction is continued, the pre-injection values of the ipsilateral flexor and crossed extensor reflexes increases, both with respect to amplitude and duration of the response.

4. After abrupt cessation of morphine or methadon, the ipsilateral flexor and crossed extensor reflexes continue to increase in magnitude, while the knee jerk and ipsilateral extensor thrusts diminish markedly. Spontaneous rhythmic activity appears in the hindlimbs (dog in lateral recumbent position) as early as 30½ hours after withdrawal of morphine and 9 hours after withdrawal of methadon. This activity increases in magnitude and frequency until a peak is reached about the 72nd.-90th. hour after morphine and about the 24th-30th hour after methadon withdrawal, following which there is a gradual return of the hindlimbs to the pre-addiction status over a period of 10-14 days. However, hyperactivity of the ipsilateral flexor reflex may persist for as long as 4 months after withdrawal of morphine.

5. After withdrawal of morphine or methadon, general signs of abstinence such as restlessness, tremors, fever, yawning, vomiting, lacrimation, rhinorrhea, and occasionally diarrhea appear at about the same time as the spontaneous activity in the hindlimbs. These general signs also reach a peak at about the same time as the hindlimb spontaneous activity, but they subside much more rapidly than the changes in reflexes which occur during the withdrawal period.

6. The effects of sodium pentobarbital, neostigmine, eserine, and elevation of body temperature on the hindlimbs of chronic spinal dogs are described.

7. The data are discussed with reference to the problems of loci of action of morphine and methadon, theories of physical dependence, and studies of physical dependence-producing liability of drugs.

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origin of the morphine or methadon abstinence syndrome in man must be tentative, pending further studies on addiction in chronic spinal monkeys or chimpanzees.

From a clinical standpoint, our findings afford further evidence that tolerance and physical dependence do develop to methadon (8, 9). In the chronic spinal dog, the methadon withdrawal syndrome is of earlier onset, somewhat more severe, and of shorter duration than that of morphine. However, it is quite likely that in chronic spinal animals we are dealing with a sensitized preparation, since Cannon and Haimovici (10) have shown that "denervated" motoneurones, just like denervated effector organs, are sensitized to chemical agents. The isolated spinal cord is "denervated" from higher segments and may therefore respond more actively to small chemical changes in the cord or to humoral agents. The severity of the withdrawal changes in the hindlimbs, therefore, may not be a reliable indication of the severity of the effects on the animal as a whole, except when used in comparison with a standard physical dependence-producing agent, like morphine.

Regardless of its role in the addiction process in intact animals or man, the spinal cord affords a convenient, relatively simple and fairly well understood sample of nervous tissue. Therefore, the changes in the hindlimb reflexes of chronic spinal dogs during addiction to morphine and methadon may be considered from the standpoint of a theory of morphine addiction. Tatum, Seevers and Collins (11) have postulated that morphine depresses some portions of the central nervous system and stimulates others. The stimulant effects outlast the depressant actions and therefore increase progressively during addiction and become manifest as withdrawal signs after cessation of injections. Our findings confirm the facts that morphine does have simultaneous depressant and excitant effects and that little or no tolerance develops to the latter. However, during withdrawal, the excitant effects (e.g., on the ipsilateral extensor thrusts) do not become manifest; in fact they become markedly depressed, while those reflexes which were depressed by morphine (spontaneous movements, ipsilateral flexor and crossed extensor reflexes) become greatly exaggerated. This is more in keeping with theories of physical dependence based on concepts of homeostatic adaptation such as those advanced by Wuth (12), and Himmelsbach (13) to explain the autonomic disturbances during abstinence from morphine. Our findings in chronic spinal dogs indicate that the somatic nervous system is also involved, perhaps in an analogous manner.

The effects of neostigmine and eserine on the hindlimb reflexes of chronic spinal dogs are included in this report because of their similarity to the changes observed during morphine or methadon withdrawal in such preparations. Since neostigmine and eserine exert their effects primarily through inhibition of cholinesterase activity, it would be of interest to investigate the cholinesterase-acetyl-choline-choline acetylase system in nervous tissue during a cycle of addiction to morphine or methadon.

The relation of the findings in the present investigations to the question of the role of psychic factors in the genesis of the morphine and methadon withdrawal syndrome has been considered elsewhere (14).

# BROMAL HYDRATE AND CHLORAL HYDRATE: A PHARMACOLOGICAL CONTRAST AND ITS CHEMICAL BASIS

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Almost immediately after the announcement of the anesthetic action of chloral hydrate by Liebreich in 1869, investigation of the analogous bromine compound was undertaken independently by several workers (1-4). At that time there was general acceptance of Liebreich's idea that chloral hydrate owes its effects to the release of chloroform in the blood, and it seemed logical to presume that bromal hydrate, which undergoes the same type of decomposition in alkaline solution *in vitro*, would produce similar pharmacological effects. However, it was found by all of these workers that bromal hydrate differs completely in its pharmacological action from chloral hydrate. Later investigations have confirmed this conclusion (5-7). All of the reports are in agreement in showing bromal hydrate to be much more toxic than chloral hydrate. The effects that have been observed in laboratory animals after the administration of bromal hydrate include strong local irritation of mucous surfaces, excitatory effects on the central nervous system, vomiting, defecation, dyspnea, salivation, and increased secretion from the respiratory tract. The anesthetic effect characteristic of chloral hydrate failed to manifest itself in the bromine analogue, although a terminal comatose state was frequently seen. On isolated tissues as well, bromal hydrate was found much more toxic than chloral hydrate (6, 8, 9). Despite the unpromising indications of the laboratory work, bromal hydrate even had a limited human trial (1, 3, 4), including an experiment performed on himself by Dougall (3). In man, as in the other animals, the drug lacked any definite hypnotic action. Moreover, some distressing symptoms were encountered, which were the counterparts of effects that had been seen in laboratory animals. Bromal hydrate has found no use in therapeutics.

The remarkable qualitative pharmacological difference between bromal hydrate and chloral hydrate has as yet received no adequate explanation. In the present investigation an attempt has been made to elucidate this problem by comparing the chemical reactions which the two compounds undergo *in vivo* and *in vitro*. The characteristic narcotic action of chloral hydrate appears to depend in large part at least on the rapid reduction of most of the compound to trichloroethanol (10). If bromal hydrate should follow the same metabolic course, the resulting tribromoethanol should produce narcotic effects quite similar to those of trichloroethanol. That bromal hydrate lacks any such simple narcotic action is indicative that its metabolic fate differs in some way from that of chloral hydrate, and herein might be expected to lie the explanation of the

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*Determination of tribromoacetic acid in plasma.* A sample of 1 cc. of plasma is added to 9 cc. of the tungstic acid solution of Van Slyke and Hawkins (18). After centrifugation, 1 cc. of the supernatant liquid is transferred to a test tube. To this are added 5 cc. of pyridine, followed by 4 cc. of 8*N* sodium hydroxide. Heating and subsequent procedures are the same as above. Plasma concentrations as low as 7 mgm. per l. are determinable.

*Determination of bromal hydrate in aqueous solutions.* To a 1 cc. sample of the aqueous solution are added 5 cc. of pyridine, then 4 cc. of 8*N* sodium hydroxide. Heating and subsequent procedures are as above. Amounts of bromal hydrate as small as 1 microgram are determinable.

*Determination of bromal hydrate in ether solution.* To 1 cc. of the ether solution is added 0.1 cc. of pyridine. The ether is then evaporated in the same manner as the pentane in the tribromoethanol method. To the residue are added 5 cc. of pyridine, then 4 cc. of 8*N* sodium hydroxide. Subsequent treatment is as above.

**SEARCH FOR TRIBROMOETHANOL AND TRIBROMOACETIC ACID AS METABOLIC PRODUCTS OF BROMAL HYDRATE.** Two dogs were given bromal hydrate intramuscularly in aqueous solution in doses of  $4 \times 10^{-4}$  moles (120 mgm.) and  $3 \times 10^{-4}$  moles (90 mgm.) per kgm., respectively. Both animals died within about half an hour. A third dog received  $8 \times 10^{-5}$  moles (24 mgm.) per kgm. intravenously in divided doses over a period of 15 min. This dog also died about half an hour after the last dose. The most prominent effects seen in all three animals were salivation, vomiting, defecation, severe dyspnea, and the appearance of large amounts of frothy liquid at the nose and mouth. Death was preceded by a brief period of coma.

Blood samples were taken at various intervals after dosage, including the period of terminal coma. At no time in any of the animals did tribromoethanol or tribromoacetic acid appear in the plasma in detectable amounts. Neither was bromal hydrate itself found in any of the plasma samples. However, because of the possibility that some of the loss of bromal hydrate could have occurred after the collection of the samples, these analyses for bromal hydrate have little significance. From its reactions *in vitro* (see below), it was judged that bromal hydrate could scarcely be expected to exist in measurable concentration for more than a very brief interval after its entrance into the circulation, and therefore no effort was made to devise a method that would prevent further disappearance of bromal hydrate following the collection of a blood sample.

In order to estimate the maximum amount of bromal hydrate that could have been converted to the alcohol and the acid, and to exclude the possibility that those hypothetical metabolic products might play any part in the effects of bromal hydrate, some experiments with tribromoethanol and tribromoacetic acid were performed on dogs. The results are summarized in the following paragraphs. The conclusions drawn from them concerning the metabolic fate of bromal hydrate are to be found in the section, "Discussion," below.

*Tribromoethanol.* A plasma level of about 100 mgm. per l. is required to produce anesthesia, and levels below 50 mgm. per l. scarcely produce noticeable effects. The minimum dose which, administered intramuscularly in aqueous solution, gives a plasma concentration detectable by the present method is  $5 \times 10^{-5}$  moles (14 mgm.) per kgm.

*Tribromoacetic acid,* like trichloroacetic acid, is found in the plasma in concen-

pharmacological differences. The chemical methods previously developed (10) for chloral hydrate, trichloroethanol, and trichloroacetic acid are applicable with minor modifications to the determination of the corresponding bromine compounds. These methods have made it possible to estimate to what extent bromal hydrate could be reduced or oxidized like chloral hydrate, as well as to study the reactions of bromal hydrate with various compounds *in vitro*.

**MATERIALS.** Bromal hydrate was prepared from bromal and water and was recrystallized from water. After drying over calcium chloride at 50 mm. Hg, the compound melted at 53.0°–53.8°C.

Tribromoacetic acid was prepared by the oxidation of bromal with fuming nitric acid, as described by Schäffer (11). After several crystallizations from heptane, the melting point was 130°–132°C.

The tribromoethanol used was the preparation marketed by the Winthrop Chemical Co. as "Avertin Crystals." The sample used melted at 78°–79°C.

**CHEMICAL METHODS. General remarks.** These methods are based on the same principles as those used previously for the analogous chlorine compounds (10). Differences in physical and chemical properties have necessitated some modifications of the procedures to adapt them to use with the bromine compounds. Since there was no occasion in the present work for the simultaneous determination of bromal hydrate and tribromoacetic acid, the procedure that was used for differentiating the corresponding chlorine compounds was not needed. The color produced in the Fujiwara reaction by bromal hydrate and tribromoacetic acid, like that produced by chloral hydrate and trichloroacetic acid, is crimson, the absorption spectra being quite similar for the bromine and chlorine compounds. Tribromoethanol, like trichloroethanol, does not itself give the Fujiwara reaction, and the method depends upon its oxidation to tribromoacetic acid.

Tribromoethanol has previously been determined in blood by a method devised by Sebening (12), which depends on the determination of bromine in an ether extract. Sebening's method with some modifications was used in a number of studies (13–17). The method described here is more sensitive and easier to perform, but there is no reason to believe that it would give different results. The pharmacological work done with tribromoethanol by use of the present method is in no essential disagreement with that reported by use of the older method.

**Determination of tribromoethanol in plasma.** The solvents used are the same as those used in the determination of trichloroethanol. A sample of 2 cc. of plasma is shaken with 12 cc. of "heptane." After centrifugation, 10 cc. of the heptane layer is removed and shaken with 16 cc. of water. After centrifugation, 15 cc. of the aqueous layer is removed. In this is dissolved 5 grams of sodium chloride, and the solution is shaken with 4 cc. of "pentane." Of the pentane layer 3 cc. is transferred to a test tube, and 0.1 cc. of pyridine added. The pentane is then evaporated as in the method for the chlorine compound. To the residue is added 0.5 cc. of a solution consisting of: potassium dichromate, 1 gram; water, 45 cc.; concentrated sulfuric acid, 55 cc. The tube is now kept at 20°C. for 30 min., after which time it is immersed in an ice bath and 4 cc. of 8*N* sodium hydroxide is added slowly with stirring. After the addition of 5 cc. of pyridine, the tube is immersed in a bath of boiling water for 1 min., during which time the contents are stirred. The tube is cooled immediately in an ice bath. A sample of the upper phase is diluted and the intensity of color measured as in the methods for the chlorine compounds. The optical density is a linear function of the concentration of tribromoethanol in the plasma.<sup>1</sup> Concentrations as low as 3 mgm. per l. are determinable.

<sup>1</sup> Distribution coefficients of tribromoethanol: "heptane"/water, 0.93; "pentane"/sodium chloride solution (300 grams per l.), 4.6. If the distribution coefficient were the same for plasma as for water, the final 3 cc. portion of pentane would contain 16 per cent of the tribromoethanol originally present in the plasma sample.

It should be noted that the removal of bromal hydrate from solution by albumin bears no resemblance to the reversible "binding" by which many other compounds are attached to albumin, but is rather an irreversible chemical reaction, and one which probably involves the rupture of bonds within the bromal hydrate molecule.

*Reaction with cysteine.* In dilute aqueous solution, at room temperature, bromal hydrate reacts very rapidly with cysteine, as evidenced by the fact that

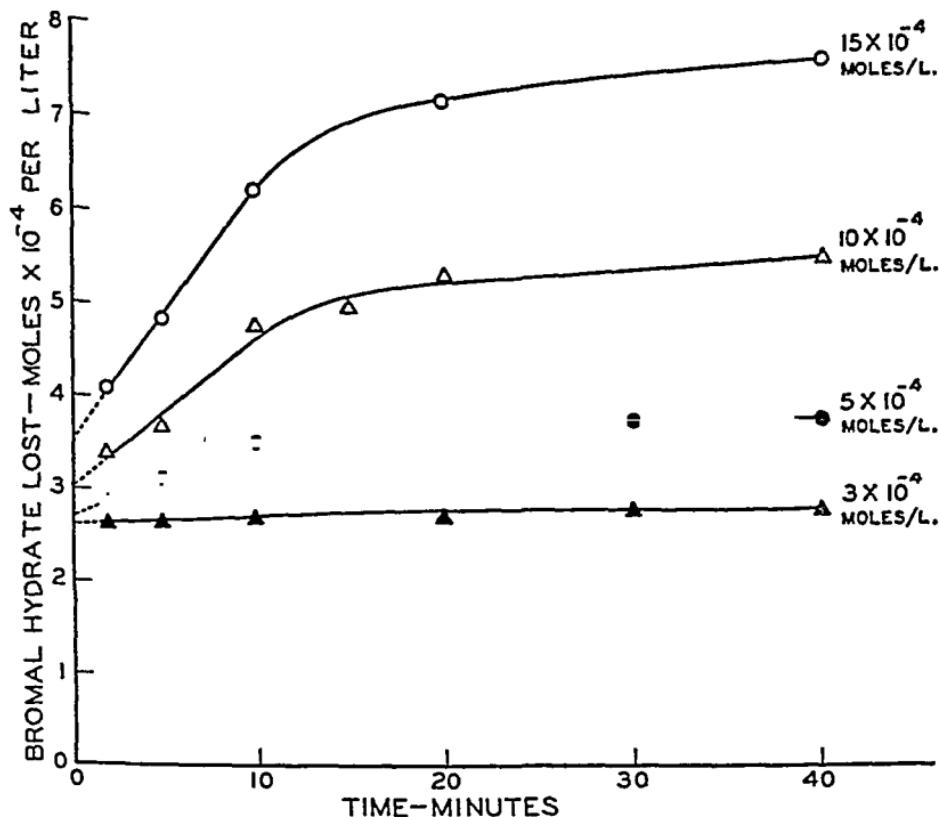


FIG. 1. REACTION OF BROMAL HYDRATE WITH HUMAN SERUM ALBUMIN, 50 GRAMS PER L. IN 0.2 M PHOSPHATE BUFFER, pH 7.4 AT 37° C

The ordinate is the amount of bromal hydrate not recoverable in the supernatant after precipitation of the protein and centrifugation. The abscissa is the time intervening between addition of bromal hydrate and precipitation of the protein. The numerical value opposite each curve indicates the total amount of bromal hydrate added, *i. e.*, the concentration that would have resulted if none had reacted with the protein.

addition of cysteine to a solution of bromal hydrate reduces the amount of bromal hydrate extractable with ether. When the reaction was carried out in 0.2 M phosphate buffer, pH 7.4, with an amount of bromal hydrate to give a calculated final concentration of 0.001 *M*, and when the mixing of the bromal hydrate and cysteine solutions was performed in such a way as to minimize the occurrence of temporary, local excesses of either reagent during the reaction, it was found that the minimal molar ratio of cysteine to bromal hydrate necessary

trations indicating that it is distributed in extracellular water. The plasma concentration falls more rapidly than does that of trichloroacetic acid. An intravenous dose of 10 mgm. per kgm. is without noticeable effect. The minimum intravenous dose that would give a plasma concentration detectable by the present method is  $7 \times 10^{-6}$  moles (2 mgm.) per kgm.

**THE CHEMICAL REACTIONS OF BROMAL HYDRATE IN VITRO.** Under conditions that can be considered physiological, bromal hydrate proved to be quite reactive with certain substances *in vitro*. Some of the chemical reactions of bromal hydrate were studied with a view to elucidate the nature of its pharmacological action.

*Disappearance of bromal hydrate from serum.* When bromal hydrate is added to plasma or serum *in vitro*, it does not all appear in the filtrate after precipitation of the proteins with any of the familiar precipitants. Neither can it all be removed by extraction of the plasma or serum with ether, even if the extraction is performed as soon as possible after the addition. The disappearance of bromal hydrate may be partial or complete, depending on the amount added. With one sample of human serum that was studied by the addition of various amounts of bromal hydrate at 37°C. and extraction with ether after 2 min., the bromal hydrate disappeared completely if it was added in amounts less than sufficient to give a calculated concentration of  $5 \times 10^{-4}$  moles (150 mgm.) per l. When chloral hydrate was added to the same sample of serum in a concentration of  $10^{-3}$  moles (165 mgm.) per l., there was no loss detectable by the methods used within 30 min. at 37°C. When bromal hydrate is added to an ultrafiltrate of serum or to a filtrate after precipitation of the proteins with tungstic acid, the loss is much smaller and occurs more slowly than is the case with serum. This suggests that the serum proteins are largely responsible for the loss of bromal hydrate. Indeed the experiments described below indicate that the reaction with albumin is sufficient of itself to account for the greater part of the disappearance of bromal hydrate from serum.

*Reaction with serum albumin.* Human serum albumin (Cohn's Fraction V) was dissolved in 0.2 M phosphate buffer, pH 7.4, to give a concentration of 50 grams per l., and various amounts of bromal hydrate were added. The rate of reaction at 37°C. was studied, advantage being taken of the fact that the reaction can be arrested by precipitation of the protein with the tungstic acid solution of Van Slyke and Hawkins (18). The results are shown in figure 1. A certain amount of bromal hydrate appears to be removed by the protein almost immediately. This amount ( $2.6-3.5 \times 10^{-4}$  moles per l.) varies relatively little with the amount of bromal hydrate added. For about the next 10 min. the rate of reaction is roughly proportional to the remaining concentration. Thereafter it becomes much slower. If the molecular weight of the albumin is assumed to be 70,000, it can be calculated that in the initial rapid reaction 1 mole of bromal hydrate is removed by 2.0 to 2.7 moles of protein. The correspondence with the minimal molar ratio of cysteine required to destroy bromal hydrate (see below) is close. If the initial reaction with protein involves the same process as that with cysteine, *viz.*, oxidation of a sulphydryl group, approximately one sulphydryl group in each albumin molecule is entering into the reaction.

cystine. The fact that almost 3 moles of cysteine can be converted to cystine by 1 mole of bromal hydrate shows that more than one C-Br bond must be capable of rupture. If the second bromine should separate in the neutral condition, it would oxidize one additional molecule of cysteine and account for the observed yield. If the second bromine should separate in the positive condition, this step must either take place to a limited extent or the bromine must take part in reactions other than the simple oxidation of cysteine to cystine. Otherwise 4 moles of cysteine should appear as cystine. The experiments described above are not sufficient to settle conclusively the question of the number of bromine atoms that are released from the bromal hydrate molecule and the charge that they carry on disruption of the bonds; yet there can be little doubt concerning the fundamental nature of the oxidative process.

An experiment in which chloral hydrate and cysteine were mixed in the buffer at pH 7.4 to give a concentration of 0.0005 M of the former and 0.005 M of the latter failed to show any significant loss of ether-extractable chloral hydrate after 30 min. at 37°C. If chloral hydrate undergoes a reaction with cysteine analogous to that of bromal hydrate, the reaction rate is of an entirely different order of magnitude. If the reaction of chloral hydrate with cysteine leading to the formation of 2-(trichloromethyl)-4-thiazolidinecarboxylic acid, as described by Schubert (19), takes place under these conditions, the compound must be dissociable on extraction with ether.

**DISCUSSION.** 1. *The metabolic fate of bromal hydrate and its lack of anesthetic action.* Although the present work failed to demonstrate any reduction of bromal hydrate to the alcohol or oxidation to the acid, the low dose that it was possible to administer precluded setting the maximum limits of the reactions at very low levels. From the data presented above, it may be estimated that the dog receiving the lower intramuscular dose of bromal hydrate could not have converted more than about 16 per cent to the alcohol. The significance of this estimate is questionable because of the possibility that absorption was incomplete at the time of death. The dog receiving the intravenous dose could not have converted more than about 60 per cent to the alcohol. Likewise, the dogs given the intramuscular doses could not have oxidized more than about 2 per cent to the acid, nor the dog given the intravenous dose more than about 10 per cent. It thus appears that at least so far as reduction to the alcohol is concerned, bromal hydrate cannot follow this metabolic path to the same extent as does chloral hydrate. The destruction of bromal hydrate by more rapid reactions would of itself appear to be an adequate factor to prevent any large amount from undergoing reactions analogous to those of chloral hydrate.

It can certainly be said that insufficient tribromoethanol is produced to play any significant role in the pharmacological effects of bromal hydrate. Consequently the lack of any anesthetic action analogous to that of chloral hydrate is understandable. By some of the early workers who were overzealously trying to find some pharmacological analogy in the structurally analogous compounds, the terminal coma sometimes produced by bromal hydrate was considered to be of the same nature as chloral hydrate anesthesia. In view of the present work, the

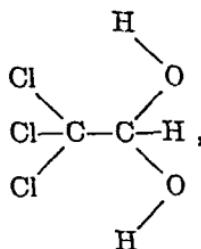
to destroy all of the ether-extractable material giving the Fujiwara reaction was about 2.2. At smaller ratios, the destruction was approximately a linear function of the ratio. No conditions were found under which 1 mole of bromal hydrate could be destroyed by less than 2 moles of cysteine. If the method of mixing favored the occurrence of excess of cysteine during the reaction, considerably larger amounts of cysteine were required to destroy 1 mole of bromal hydrate.

The fact that similar experiments gave no evidence of reaction between bromal hydrate and glycine suggested that the reaction is with the sulphydryl group of cysteine. Furthermore the fact that bromal hydrate is capable of slowly oxidizing the iodide ion to iodine at pH 7.4 suggested that the reaction with the sulphydryl group is an oxidation. That oxidation of the sulphydryl group does occur can be demonstrated by the isolation of cystine as a product if the reaction is carried out in sufficiently concentrated solution. In the buffer at pH 7.4, cystine separates from solution if the amount of bromal hydrate added is sufficient to give a calculated concentration greater than 0.004 M and cysteine is an excess.

An estimate of the yield of cystine was made in the following experiment. To 12 cc. of oxygen-free 0.2 M phosphate buffer, pH 7.4, was added 0.004 moles of L-cysteine hydrochloride plus an equivalent amount of sodium hydroxide. With this solution was mixed a solution of 0.0004 moles of bromal hydrate in 4 cc. of the buffer. The white precipitate, which began to separate almost immediately, was filtered off and washed with water, methanol, and ether. The filtrate gave a positive nitroprusside test. After drying over phosphorus pentoxide and sodium hydroxide, the precipitate weighed 141 mgm. It had a very low solubility in water but dissolved readily in 1 N hydrochloric acid or 1 N sodium hydroxide. The alkaline solution gave a negative nitroprusside test, but if first treated with a little sodium cyanide, gave a strongly positive nitroprusside test. The yield as cystine was 0.000588 moles. Each mole of bromal hydrate had converted 2.94 moles of cysteine to cystine. In a similar experiment in which the molar ratio of cysteine to bromal hydrate was 2, 70 per cent of the cysteine was recovered as cystine, and the filtrate gave a negative nitroprusside test.

Although other reactions may also take place, the predominant primary reaction between bromal hydrate and cysteine is the oxidation of the sulphydryl compound to the disulfide. This oxidizing action of bromal hydrate resembles that of other "positive halogen" compounds, and it almost certainly results from the "abnormal" rupture of C-Br bonds in such a way that the carbon atom retains one or both of the two electrons shared in the covalent bond. In the former case bromine would separate from carbon in the neutral condition and could acquire one electron to become a bromide ion. In the latter case bromine would be positively charged and could acquire two electrons to become a bromide ion. The fact that 1 mole of bromal hydrate cannot be destroyed with less than 2 moles of cysteine suggests that the first step in the oxidative reaction is brought about by the separation of one bromine in the positively charged condition. It could thus by acquisition of 2 electrons oxidize 2 molecules of cysteine to 1 of

Another factor that may be of great importance in determining the relative reactivities of the halogens is the equilibrium proportion of carbonyl and hydrated, or *gem*-diol, forms. Davies (23) has attributed the peculiar stability of the hydrate of chloral to the interaction of the C-Cl and O-H dipoles. This leads to the configuration,



in which the OH groups are clamped in a position corresponding to maximum interaction with the adjacent dipoles, and the H is not free to rotate about the C-O bond. In this form the molecule cannot eliminate water or react as an aldehyde. Davies' calculations show a frequency distribution of  $2.9 \times 10^6 : 1$  in favor of this configuration against that in which both hydroxyls are oriented away from the chlorine and toward each other.

In bromal hydrate the halogen is less strongly negative than in chloral hydrate. The force to which Davies attributes the stabilization of the *gem*-diol form will accordingly be less. A greater proportion should have hydroxyl groups free to rotate, and thus a greater proportion should be in the carbonyl form. Indeed Davies found some indication of absorption by bromal hydrate at  $2.76 \mu$ , the wavelength characteristic of a freely rotating hydroxyl. With chloral hydrate there was no indication of an absorption band at that wavelength. Another piece of experimental evidence that might be considered indicative of a higher proportion of carbonyl form in the bromine compound is the fact that bromal hydrate gives the Schiff test for aldehydes, whereas chloral hydrate does not.

The activating influence of a carbonyl group on the carbon-halogen bonds would be expected to be greater than that of two hydroxyl groups. If the angles between the moments and the C-C axis are at all near the values that have been given for chloral (24) and chloral hydrate (23), a C=O moment of 2.8 D should exert a greater polarization effect than two C—O moments of 0.8 D each. Accordingly, in reactions of the type studied here, the carbonyl form would be expected to be the more reactive species. The high reactivity of a halogen attached to a carbon atom alpha to a carbonyl group is well known.

The high reactivity of bromal hydrate and the low reactivity of chloral hydrate may then be attributable not only directly to the inherent differences between the properties of the C-Br and C-Cl bonds, but also to the fact that bromal hydrate can furnish a molecular form containing a more powerful polarizing group.

4. *The pharmacology of analogous compounds of chlorine and bromine.* Most of

two phenomena can scarcely be thought to have the same physiological basis. Dougall (3), one of the original workers with bromal hydrate, offered a discerning appraisal of its effect: "The bromal hydrate had undoubtedly lulled sensation, but it was by permanently destroying it."

The work of Maraldi (20) has long been cited as showing that bromal hydrate, like chloral hydrate, undergoes reduction in the body to the alcohol. It was indeed Maraldi's conclusion that bromal hydrate is excreted entirely in the form of urobromalic acid, a conjugation product of tribromoethanol with glucuronic acid. Yet he did not claim isolation of the conjugation product, nor does his report present any evidence that can be considered at all convincing that tribromoethanol was in the urine in any conjugated form. Urobromalic acid was undoubtedly not actually known prior to its isolation (as a metabolic product of tribromoethanol) by Endoh (21) in 1924. From the reactions that bromal hydrate undergoes *in vitro*, it appears probable that a considerable proportion, if not all, of the bromine of the compound must leave the body as the bromide ion.

2. *The nature of bromal hydrate toxicity.* Although work of the type reported here cannot be expected to point conclusively to the mechanism by which the drug causes the death of an animal, it does furnish some indications that are at least suggestive. A compound that reacts so rapidly with sulphydryl groups under physiological conditions would be expected to inactivate the various enzymes the activity of which has been shown to depend upon free sulphydryl groups. Although this mechanism may be sufficient to account for the toxic effects of the compound, it is conceivable that biochemical or even physical disturbances in the cell resulting from other reactions might be of greater importance. At any rate, it is difficult to believe that the high toxicity of bromal hydrate is not intimately connected with the high reactivity of the halogen in the molecule.

3. *The structural basis of the reactivity of bromal hydrate.* The pharmacological difference between bromal hydrate and chloral hydrate appears then fundamentally to depend upon the ability of the former substance, and the inability of the latter, to enter into "positive halogen" reactions under physiological conditions. This difference in reactivity of halogen in the two compounds is explicable, in a general way at least, in terms of the known structural properties of the molecules. As explained by Waters (22), "positive halogens" result when the induced electrostatic energy, due to an electrical field of vector orientation opposing that of the carbon-halogen linkage, is greater than the intrinsic electrostatic energy of this same linkage. In any two analogous compounds of chlorine and bromine, the properties of the carbon-halogen bonds are such as to favor the production of positivity in bromine as compared with chlorine. The electrostatic energy of the C-Br bond is less than that of the C-Cl bond. Moreover, the polarizability of the C-Br bond is greater than that of the C-Cl bond. Thus the dipole moment of a C-Br bond will be influenced to a greater extent than will that of an analogous C-Cl bond either by a dipole situated elsewhere in the same molecule or by a dipole in a reagent molecule.

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the pairs of analogous organic compounds of chlorine and bromine that have hitherto been studied have shown little qualitative difference in their pharmacological actions, and the feeling is probably prevalent in pharmacological thought that this is the relationship generally to be expected. A typical example of this sort is furnished by trichloroethanol and tribromoethanol, compounds which both produce anesthesia and which differ principally quantitatively with respect to their effective molar concentrations. In pairs of analogous halogen compounds that show no striking qualitative difference in pharmacological action, the halogen is not reactive under physiological conditions in either member of the pair, or at least not so reactive as obviously to dominate the pharmacological picture. The situation with respect to chloral hydrate and bromal hydrate is in marked contrast. These two compounds are remarkable in that neither appears to exert its characteristic pharmacological effect as a consequence of its physical presence as such in the body, but rather as a result in each case of the compound undergoing chemical reactions, the reactions being totally different in the two cases. The intramolecular electrostatic forces in these molecules lie in a critical range, where the changes accompanying the replacement of chlorine by bromine are sufficient to alter tremendously the probability of the molecule entering into a chemical reaction.

The author is indebted to Dr. Leslie Hellerman for valuable advice and suggestions concerning the study of the chemical reactions of bromal hydrate.

#### SUMMARY

1. When bromal hydrate is administered to dogs, neither tribromoethanol nor tribromoacetic acid can be demonstrated in the plasma.
2. Under physiological conditions *in vitro* bromal hydrate reacts rapidly with serum albumin and with cysteine, the latter substance being oxidized to cystine.
3. It is suggested that the "positive halogen" properties of bromal hydrate are responsible for its high toxicity.
4. The structural features responsible for the difference in reactivity of bromal hydrate and chloral hydrate are discussed.

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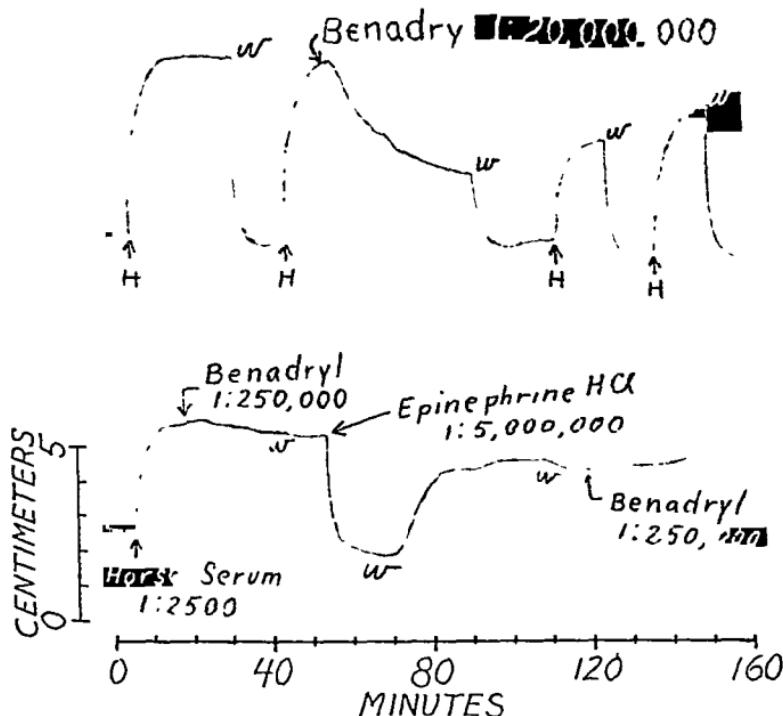


FIG. 1. THE RESPONSES OF THE SENSITIZED TRACHEAL CHAIN TO (H) HISTAMINE PHOSPHATE 1:500,000 AND TO THE ANTIGEN (HORSE SERUM 1:2500)

(W) indicates washing. Upper tracing shows the effect of Benadryl on the histamine spasm. Lower tracing shows the effect of Benadryl and epinephrine on the anaphylactic spasm.

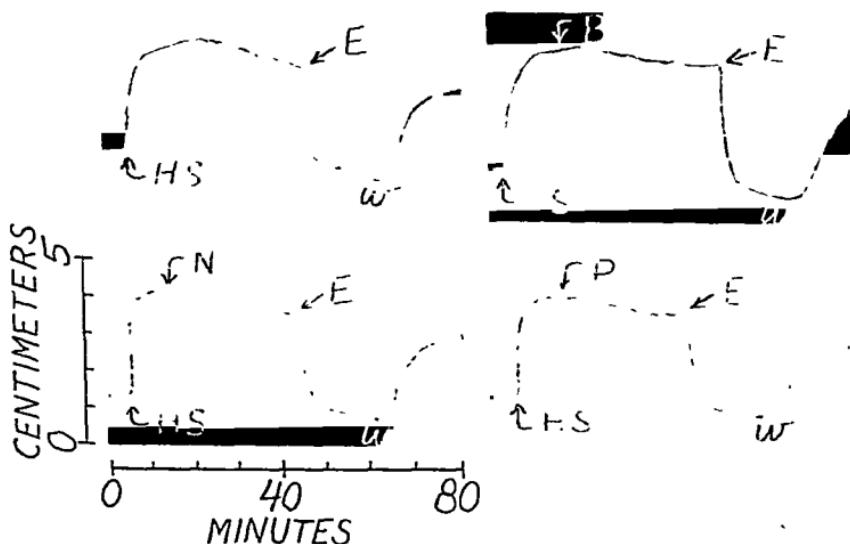


FIG. 2. THE EFFECT OF (B) BENADRYL, (N) NEO-ANTERGAN, (P) PYRIBENZAMINE, EACH IN A CONCENTRATION OF 1:250,000, AND (E) EPINEPHRINE 1:5,000,000 ON THE SPASM PRODUCED BY (HS) HORSE SERUM ON TRACHEAL CHAINS MADE FROM GUINEA PIGS 15 TO 17 DAYS AFTER THE SENSITIZING INJECTION

(W) indicates washing.

## THE TRACHEAL CHAIN

### II. THE ANAPHYLACTIC GUINEA PIG TRACHEA AND ITS RESPONSE TO ANTIHISTAMINE AND BRONCHODILATOR DRUGS<sup>1</sup>

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In a recent publication (1) a method was presented for the study of bronchodilator and spasmolytic drugs on the tracheal musculature of the guinea pig. In that paper a technique was described for magnifying and recording the constrictions and dilatations of the excised trachea when exposed to drugs. Briefly, the technique consists in sectioning the entire trachea of a guinea pig into twelve rings of approximately equal thickness and connecting the rings in chain-fashion with short loops of thread. The "tracheal chain", as the preparation is called by the authors, is mounted vertically in a tissue chamber containing the bathing fluid and fastened to a light muscle lever so that the simultaneous constrictions and dilatations of the twelve rings in the chain can be recorded.

The tracheal chain has been found also very useful in the study of histamine and antihistamine drugs. The results of these experiments were presented in another communication (2). The tracheal chain was shown to respond to histamine with prompt contractions which were sustained at the same level for a long period of time, thus facilitating the qualitative and quantitative study of the ability of the antihistamine drug to relieve the spasm. One of the outstanding features shown was that the tracheal chain differentiates between a bronchodilator drug and a truly specific antihistamine substance.

The present communication deals with the anaphylactic tracheal chain and its response to antihistamine and bronchodilator drugs.

*Method.* Guinea pigs were sensitized to horse serum by the intramuscular injection of 0.5 cc. of fresh normal serum. Tracheal chains were made from these animals 15 to 22 days after the injection and mounted individually in a bath of Van Dyke-Hastings' solution (3) maintained at 37.5°C. and modified to contain 0.05 per cent dextrose. The chain was connected to a light, sensitive muscle lever yielding twelve-fold magnification; the only tension used was that needed to keep the preparation in a vertical position. Satisfactory tension was obtained by following the procedure for mounting as outlined in the original paper (1).

In studying the nature of the response of the sensitized tracheal chain to the antigen, we studied also its response to histamine and observed the behavior of both responses to antihistamine and bronchodilator drugs.

**RESULTS. The Response of the Sensitized Tracheal Chain to the Antigen.** As illustrated in figures 1 and 2, tracheal chains made from guinea pigs sensitized to horse serum were found to respond to low concentrations of the antigen with prompt contractions which, like those obtained with histamine, were maintained

<sup>1</sup> Presented in part at the meeting of the Federation of American Societies for Experimental Biology held at Atlantic City, New Jersey in March 1948.

Figure 3 shows that other bronchodilator drugs, such as aminophylline and one of our experimental compounds which has been found to have definite bronchodilator activity, relax the contraction produced by the antigen on the sensitized tracheal chain. The fact that the anaphylactic spasm reappears after the epinephrine and aminophylline are removed from the bath, indicates that the temporary relaxation is merely the result of a drug acting in an opposite direction.

**DISCUSSION.** Ever since Dale and Laidlaw (4) first presented the histamine theory of anaphylactic poisoning, it has been the favored concept among most investigators that the major mechanism of the antigen-antibody mediation of anaphylaxis and allergy involves abnormal liberation of histamine. The results presented in this paper stir up interesting speculations as to the role that histamine really plays in the production of the anaphylactic spasm in the tracheal chain.

A possible explanation for the failure of the antihistamine drugs to alleviate the anaphylactic spasm of the tracheal chain is suggested in the theory recently postulated by Dale (5), that the reaction produced when sensitized plain muscle encounters the antigen may be the result of "intrinsic histamine". Since in the sensitized tracheal chain we are dealing with isolated smooth muscle, it is possible that this internally-liberated histamine is not reached by the antihistamine present in the surrounding bathing fluid.

The finding that the anaphylactic spasm, as produced in the guinea pig trachea, is not relieved by antihistamine drugs but is relieved by bronchodilator drugs, is in agreement with the clinical findings (6-8) that, although antihistamine drugs are of definite therapeutic value in many allergic conditions, they are not effective in the relief of certain types of allergic asthma, where bronchodilator drugs are effective.

#### SUMMARY

1. Tracheal chains made from guinea pigs sensitized to horse serum respond to relatively low concentrations of the antigen with prompt contractions which are maintained at the same level for a long period of time.
2. These anaphylactic spasms are not relaxed by antihistamine drugs but are relaxed by bronchodilator drugs.
3. The anaphylactic spasm, as produced in the guinea pig trachea, differs from that produced by histamine, not only in its resistance to antihistamine drugs but also in the fact that the anaphylactic spasm cannot be relaxed by washing.

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at the same level for a long period of time. The best anaphylactic spasms were obtained with tracheal chains made from guinea pigs 15 to 17 days after the intramuscular injection of horse serum. Tracheal chains made from the sensitized animals at later days (see figure 3), were found to respond to the antigen with contractions which, although prompt, were not sustained.

The anaphylactic spasm was found to differ from that produced by histamine (see figures 1 and 3), in that the histamine contraction could be relaxed by simple washing and also could be reproduced, whereas the anaphylactic spasm could not be washed off and consequently could not be reproduced. As shown in figure 3, even though the spasm produced by the antigen was weak, it could not be relaxed by washing nor was it enhanced by the addition of a second dose of horse serum.

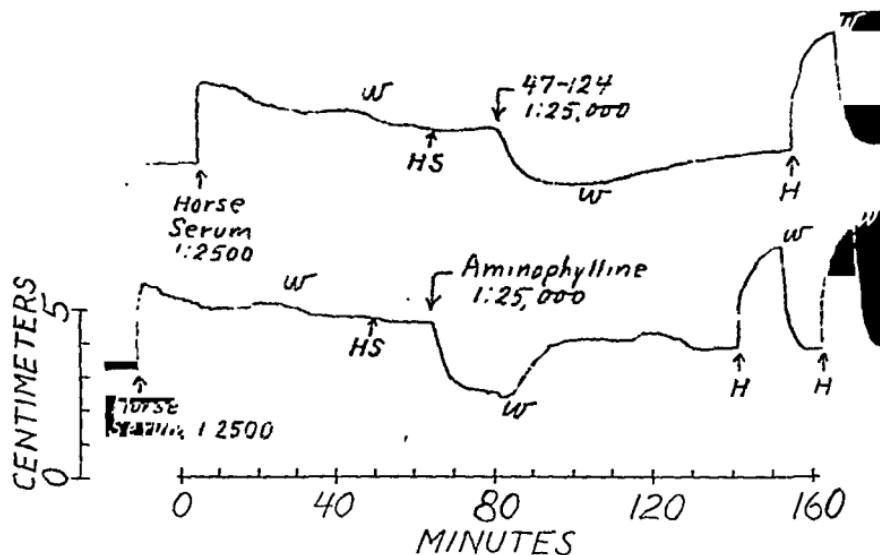


FIG. 3. THE ANTIGENIC RESPONSE OF TRACHEAL CHAINS MADE FROM GUINEA PIGS 22 DAYS AFTER THE SENSITIZING INJECTION OF HORSE SERUM

The effect of aminophylline and an experimental bronchodilator drug (47-124) is also shown, as well as the responses of the preparations to (H) histamine phosphate 1:500,000. (W) indicates washing.

*The Effect of Antihistamine and Bronchodilator Drugs on the Anaphylactic Spasm.* Figures 1 and 2 show that the spasm produced by the antigen on the anaphylactic tracheal chain is not relieved by relatively high concentrations of highly active antihistamine drugs such as diphenhydramine (Benadryl), tripeleannamine (Pyribenzamine) and pyranisamine (Neo-antergan), even when allowed to act for a long period of time. However, a good bronchodilator drug such as epinephrine, causes a prompt relief. In sharp contrast, the upper tracing of figure 1 shows that the marked spasm induced by histamine can be relaxed by a relatively low concentration of Benadryl. The smaller contractions obtained with subsequent equal doses of histamine, indicate the slow recovery of the preparation from the effect of the antihistamine drug.

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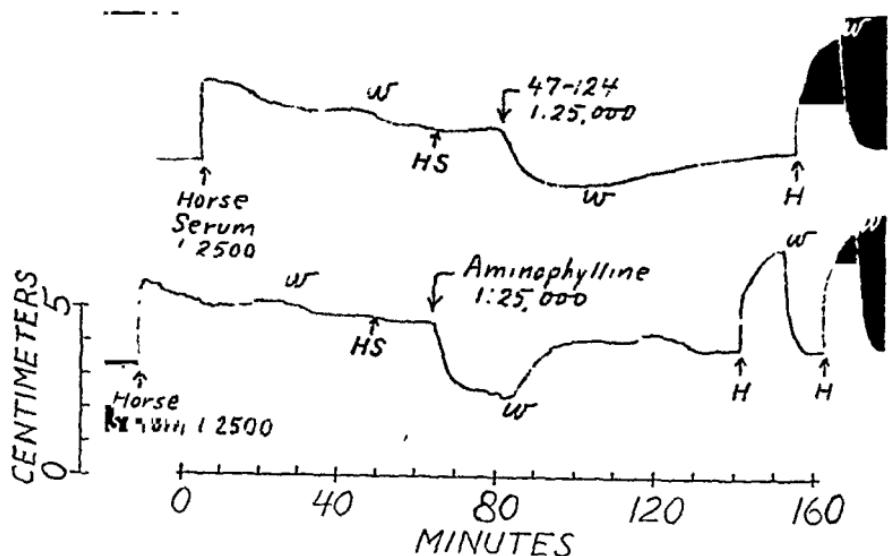
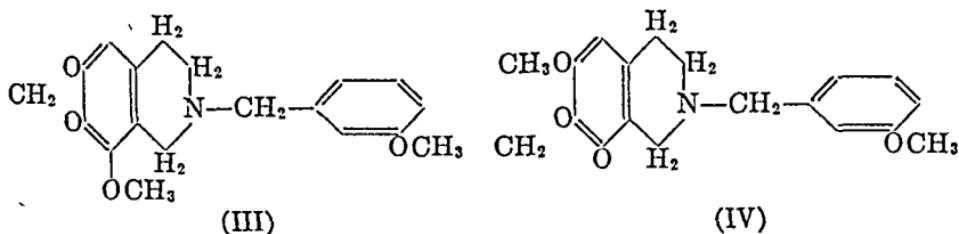


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Compounds (II), (III) and (IV) were synthesized in our laboratories by C. Ernst Redemann, Burnett B. Wisegarver and Roland N. Icke, and the details will be published elsewhere (7). Assignment of the 2-(metamethoxybenzyl)-6,7-methylenedioxy-8-methoxy-1,2,3,4-tetrahydroisoquinoline structure (III) for the isomer having the higher melting hydrochloride and the 2-(metamethoxybenzyl) - 6 - methoxy - 7,8 - methylenedioxy-1,2,3,4 - tetrahydroisoquinoline structure (IV) for the isomer having the lower melting hydrochloride was made upon the basis of the work of Salway (8) on the synthesis of cotanine.

**EXPERIMENTAL.** The three compounds having the structures given were used in the form of their hydrochlorides. The secondary amine lacking the N-methyl group of compound (II) was also available from the synthesis work in the form of its hydrochloride, and was used to discover if there were any specific effect of the N-methyl group or the tertiary amine function of compound (II). The  $\alpha$ -fagarine hydrochloride used was prepared in our laboratory from *Fagara coca* leaves and was recrystallized from ethanol. The product used had a melting point of 159–160°, and gave elementary analyses corresponding to  $C_{21}H_{23}NO_5 \cdot HCl \cdot C_2H_5OH$  and a molecular weight of 452.

*Inhibitory Effects on Isolated Auricle.* While quinidine and like compounds act to depress cardiac function generally, their effect on the auricle is of primary therapeutic interest. This type of activity appears to be satisfactorily valued by the method of Dawes (9, 10). After considerable experimentation we have found guinea pig preparations to be as suitable as those from the rabbit.

**METHOD.** With certain modifications of apparatus (figure 1), the method of Dawes was used. The auricles were carefully dissected from a guinea pig heart and suspended in a 29°C. bath of oxygenated Locke solution containing per liter: NaCl 9.0 grams, KCl 0.42 grams, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.48 grams, NaHCO<sub>3</sub>, 0.30 grams, dextrose 1.0 gram, with pH adjusted to 7.5. The lower tip of the auricle was fixed in the orifice of a small bulb which formed a removable cap which was placed over the end of the U-shaped glass tube carrying the single Nichrome electrode. Oxygen was passed through the tube so that it escaped into the bath through the orifice nearly obliterated by the tip of the auricle. In this way the entire preparation was immersed in the Locke solution with a direct suspension from the recording lever without short-circuiting the electrodes. The single electrode made contact with the lower tip of the auricle and was kept at zero potential by direct connection to the grounded chassis of the stimulator. The bath served as the anode and was connected to the stimulator output. The stimulator used gave rectangular pulses of 3 milliseconds duration at frequencies continuously variable from 120 to 900 shocks per minute.

After allowing 30 minutes for equilibration of the preparation, the threshold intensity for stimulation was determined at a frequency of 170 shocks per minute, and the stimulator output voltage was set for the experiment at ten times the threshold value. The basal maximum frequency maintaining a 1:1 ratio between stimulus and response was determined

# COMPARATIVE ACTIONS OF CERTAIN COMPOUNDS LIKE ALPHA-FAGARINE

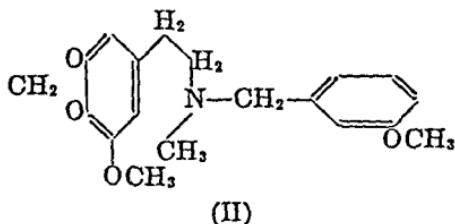
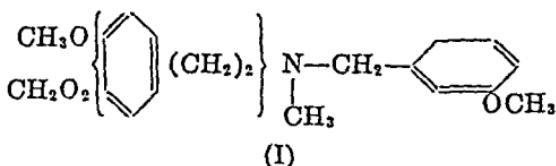
GORDON A. ALLES AND CHARLES H. ELLIS

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Studies by Stuckert (1) have particularly well defined three major alkaloids present in the South American plant *Fagara coco*. The one designated as  $\alpha$ -fagarine is a base of a chemically different type from the  $\beta$ - and  $\gamma$ -fagarines. The latter bases have been since established as the dimethoxy and monomethoxy derivatives of dictamine, and both compounds have its methoxyfuroquinoline structure (2, 3). Under the name of skimmianine,  $\beta$ -fagarine has been studied by Honda (4), with regard to certain of its pharmacological actions.

Deulofeu and co-workers (5, 6) have proposed (I) as a provisional structure for  $\alpha$ -fagarine, the position of the methoxy, methylenedioxy and methylene groups being unestablished. Interest in the problem of its structure has been increased by the recent publication of a short note (6) which reported the successful therapeutic use of the compound in six patients manifesting auricular flutter or fibrillation. It was of interest to synthesize one of the possible compounds of the general structure (I) in order to compare its pharmacological properties with those of  $\alpha$ -fagarine and other depressants of the cardiac mechanisms.



From the work of these investigators it appeared that the metamethoxybenzylamine part of the structure was established. The most common relationship of the other groupings in naturally occurring alkaloids is that of homomyristicylamine. Among commonly occurring alkaloids corresponding tetrahydroisoquinoline structures are often found, as in the mescal tetrahydroisoquinoline alkaloids anhalonine and lophophorine. It was of interest, therefore, to compare the pharmacological properties of the tetrahydroisoquinolines (III) and (IV) with those of the open chain compound (II).

All of the compounds of table 1 caused a moderate reduction in the spontaneous rhythm of the auricles, but this was not satisfactorily quantitated. Quinidine

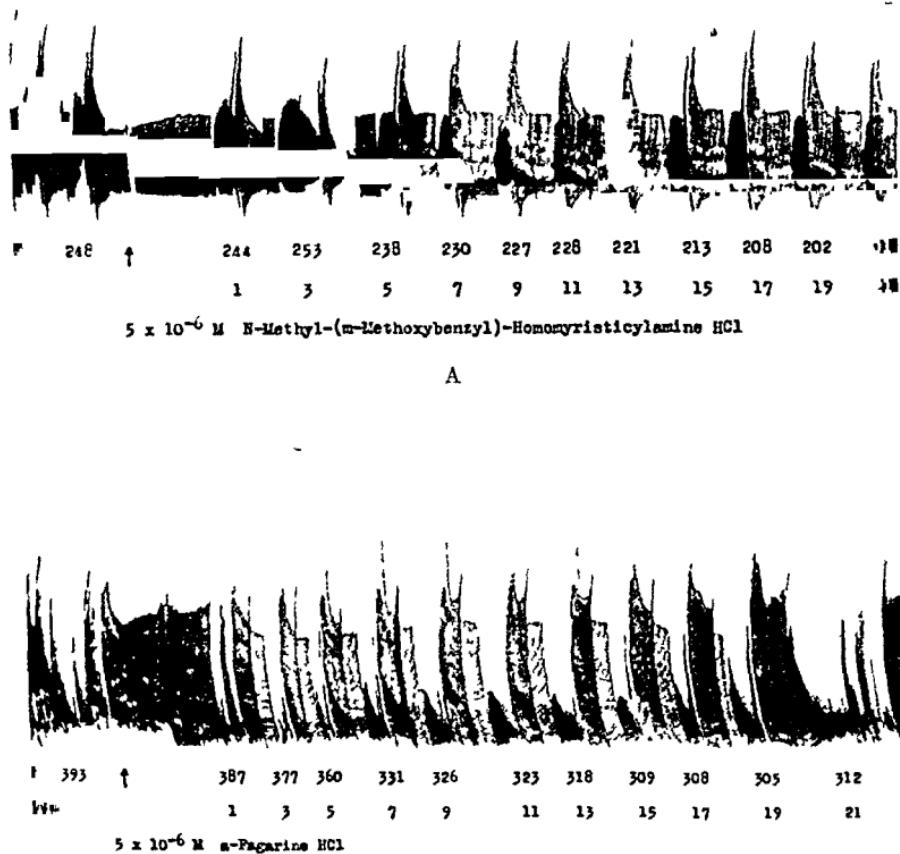


FIG 2 RESPONSE OF ISOLATED GUINEA PIG AURICLE TO  $5 \times 10^{-6}$  MOLAL *N*-METHYL (*m*-METHOXYBENZYL)-HOMOMYRISTICYLAMINE HCl (A), AND TO  $\alpha$  FAGARINE HCl (B)

The figures in line F represent the frequency of stimulation in shocks per minute at the moment of the first dropped beat, as indicated by the marked increase in amplitude of the next beat. Stimulation was discontinued at that time, and the drum was stopped between determinations. Time is in minutes after introduction of the drug into the bath.

sulfate and papaverine hydrochloride were chosen as reference standards for the above comparisons.  $\alpha$ -Fagarine appears to be quite as active a depressant of the guinea pig auricle as is quinidine, and several times more active than papav-

by gradually increasing the stimulation frequency up to the rate at which the auricle began to drop beats, as indicated by the greater amplitude of the next contraction (figure 2). Appropriate dosage of drug was then added to the bath and determinations of the maximum frequency were made at the end of 1 minute and at 2 minute intervals thereafter through 21 minutes.

Dosage with any of the compounds caused an unpredictable reduction of sensitivity to the same or other compounds. Since this reduction in sensitivity did not disappear within an hour after repeated washing with fresh Locke solution, it limited the usefulness of each

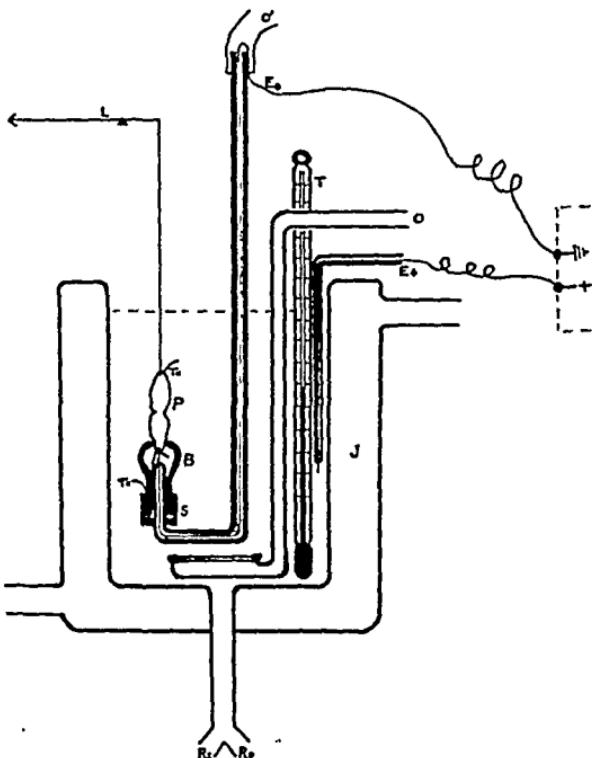


FIG. 1. SCHEMATIC ARRANGEMENT OF APPARATUS

B-glass bulb with 2 mm. orifice in tip. E<sub>+</sub>, E<sub>-</sub>—electrodes. J—heating jacket warmed by circulation of water from constant temperature bath. L-recording lever. O'-oxygen forced through U-tube to keep bulb free of Locke solution. P—isolated auricle preparation. Ri—inlet from Locke solution reservoir. Ro—outlet from bath. S—rubber sleeve to anchor lower tip of preparation and to hold bulb in position. Th—silk thread. T—thermometer.

preparation to a single dose of a single compound. Three separate preparations for each dosage of each compound were used in the experiments reported in the present study.

The relative activities of the compounds at two dosages are presented in table 1. In each case the observed maximum frequency was expressed as a per cent of the basal value, and the reduction in maximum frequency determined by subtraction of this from 100 per cent. Each figure represents the average of the three preparations used. For comparison, the mean per cent reduction during the 21-minute test period was calculated and is given in the last column of table 1.

to  $10^{-4}$ . At concentrations from  $5 \times 10^{-4}$  to  $10^{-3}$  molal, however, contraction was the usual response, with mixed effects commonly noted in the range of  $10^{-4}$  to  $5 \times 10^{-4}$  molal. The contraction, or stimulant, effects of  $\alpha$ -fagarine evident in the higher concentration range were not affected by pretreatment with a 1/10 to 1/2 mol ratio of atropine.

The synthetic compounds were similar in that both types of activity were found at appropriate concentration ranges, but their quantitative activities were different. Metamethoxybenzyl homomyristicylamine was depressant at  $2 \times 10^{-5}$  molal and more actively depressant than  $\alpha$ -fagarine in this concentration and up to  $5 \times 10^{-4}$  molal. At this higher concentration, and on occasion even at  $10^{-4}$  molal, some stimulant activity of the metamethoxybenzyl homomyristicylamine was to be observed. The N-methyl derivative (II) was more actively stimulant, being about 5 times more active in this respect than  $\alpha$ -fagarine. The isoquinoline isomers (III and IV) produced relaxation at about  $10^{-6}$  molal, but showed stimulant activity at higher concentrations. This stimulant activity was comparable to that of the N-methyl derivative (II).

In contrast to the mixed effect of  $\alpha$ -fagarine and the synthetic compounds, which were dependent on the concentration ranges used, the effects of quinidine and papaverine were observed to be depressant only, and caused relaxation in all the concentration ranges that had been studied with the other compounds. At low concentrations of about  $2 \times 10^{-5}$  molal, quinidine and papaverine were about as actively depressant as metamethoxybenzyl homomyristicylamine. At higher concentrations of about  $5 \times 10^{-4}$  molal, quinidine and papaverine were actively relaxing in their action, while metamethoxybenzyl homomyristicylamine exhibited slight stimulant action, and  $\alpha$ -fagarine was definitely stimulant in its action.

The amplitude of the spontaneous contractions of the ileum is reduced by quinidine and papaverine, and usually by metamethoxybenzyl homomyristicylamine. After  $\alpha$ -fagarine in low concentrations, the amplitude of such rhythmic contractions is usually reduced, but in higher concentrations, particularly with a moderate increase in tone, the amplitude of the spontaneous contractions may be markedly increased. With the compounds (II), (III) and (IV) the amplitude of spontaneous contractions is usually markedly increased.

It was desirable to value approximately the extent to which the compounds of interest exhibit antagonism to the stimulant effects of acetylcholine and histamine on guinea pig ileum. The direct effect of these compounds on the ileum is considerable and complicates quantitative conclusions, but an estimate of such antagonistic activities could be made. Quinidine and papaverine at  $10^{-4}$  molal produced notable diminution of response to acetylcholine at  $10^{-5}$  molal, while  $\alpha$ -fagarine was almost inactive under the same concentration ratio conditions. Metamethoxybenzyl homomyristicylamine, its N-methyl derivative (II) and the isoquinoline isomers (III) and (IV) were about as active as papaverine in antagonizing acetylcholine under the same conditions.

Against histamine, the synthetic compounds were somewhat different in their relative antagonistic activities. Using  $10^{-4}$  molal concentrations of the com-

erine, in the lower dosage range studied. Comparison at the lower dosage range appears to be the more useful, for it is notable that the reduction in maximum frequency at the higher dosage range is similar for all the compounds studied. This would indicate that a sufficient amount of almost any compound of these

TABLE 1

*Comparison of Compounds on Maximum Frequency of Stimulation Which Can Be Followed by Isolated Auricle*

(Figures are per cent reduction from initial maximum frequency)

COMPOUND	CONCENTRATION (M X 10 <sup>-5</sup> )	MINUTES AFTER INTRODUCING DRUG										MEAN REDUCTION	
		1	3	5	7	9	11	13	15	17	19		
$\alpha$ -Fagarine HCl	1.0	1.9	11.8	18.4	21.9	23.1	25.9	27.2	28.7	28.8	32.4	33.5	23.1
	2.5	8.8	10.9	20.3	27.6	31.6	38.4	37.8	38.8	41.6	44.3	44.4	31.3
Metamethoxybenzyl homomyristicylamine HCl	1.0	5.6	11.2	15.8	19.0	20.0	22.7	22.8	24.7	25.5	26.5	27.0	20.1
	2.5	4.6	10.4	18.3	23.4	28.3	31.4	33.5	37.7	41.0	44.1	46.9	29.1
N-Methyl metamethoxybenzyl homomyristicylamine HCl (II)	1.0	5.5	10.3	12.8	12.3	9.7	14.8	17.0	18.6	22.1	24.4	22.9	15.5
	2.5	9.4	14.2	20.0	30.0	23.4	7.35	3.35	9.37	4.39	1.40	5.41	30.8
2-(Metamethoxybenzyl)-6,7-methylenedioxy-8-methoxy-1,2,3,4-tetrahydrossoquinoline HCl (III)	1.0	3.5	7.8	12.3	13.6	16.1	19.2	22.4	22.4	23.8	24.9	25.8	17.4
	2.5	2.9	10.2	18.0	22.9	25.0	29.0	31.2	33.8	36.9	38.6	40.8	26.3
2-(Metamethoxybenzyl)-6-methoxy-7,8-methylenedioxy-1,2,3,4-tetrahydrossoquinoline HCl (IV)	1.0	5.4	4.9	6.2	9.2	11.7	12.5	15.0	17.1	18.8	20.2	20.8	12.9
	2.5	8.1	14.9	21.7	26.3	31.8	35.9	39.1	41.0	44.0	46.0	48.2	32.5
Quinidine · $\frac{1}{2}$ H <sub>2</sub> SO <sub>4</sub>	1.0	5.8	8.2	13.5	17.5	19.5	22.5	25.7	26.3	26.3	28.3	28.6	20.2
	2.5	10.1	16.3	22.9	29.2	31.9	34.6	37.1	38.4	41.5	42.9	43.9	31.7
Papaverine HCl	1.0	1.6	2.9	5.5	6.9	5.7	7.1	7.0	9.3	10.2	10.4	10.7	7.0
	2.5	4.8	10.3	16.4	22.9	27.8	33.2	34.7	37.7	39.1	40.4	40.8	28.0

general types can produce a mean reduction in maximum frequency of about 25-30 per cent.

*Effects on Isolated Ileum.* Ileum strips from the guinea pig were suspended in oxygenated Locke solution of the same composition as that used for the auricle preparations, but the bath was kept at 37°C.  $\alpha$ -Fagarine caused relaxation of the muscle at low concentrations, in the range of molar concentrations from 10<sup>-5</sup>

marked depression, slowing of respiration, and a few clonic twitchings prior to death.

**DISCUSSION.** In weighing the import of a reduction by a drug in the maximum frequency of electrical stimulation to which an isolated auricle preparation can respond with a 1:1 stimulus-response ratio, it is imperative that the physiological events which result in such a reduction should be considered. Dawes (10) considers that inasmuch as it involves measurement of the maximal rate, his method depends principally on refractory period. De Elfo (13) further pointed out that it serves as a measure, not of the absolute refractory period (the length of time before the muscle will again respond to a stimulus of any intensity), but of the effective refractory period (the length of time before the muscle can again transmit a propagated wave to any distance). As it has been used, it would appear to be the effective refractory period which is measured by the isolated auricle preparation.

Quinidine is considered to stop auricular fibrillation principally by prolonging the effective refractory period of auricular muscle both directly and by abolishing vagal tone, although it also depresses conduction in the junctional tissues (14).  $\alpha$ -Fagarine has been shown by Moisset de Espanés to have similar actions (15). In so far as the action is of the former type, the isolated auricle preparation should be of value in deciding whether a compound might be expected to prevent auricular flutter or fibrillation. Wegria and Nickerson (16) found that quinidine, papaverine, and procaine, compounds which lengthen the refractory period generally, raise the fibrillation threshold in dogs. The threshold was not only significantly raised, but was maintained for a significant length of time.

Even a lengthening of the refractory period is doubtless due to a generalized depression of cardiac tissue. Moisset de Espanés (17) demonstrated with respect to  $\alpha$ -fagarine, that it is more active in raising the chronaxie of the myocardium of the toad than is quinidine. He found (18) that, within the useful dosage range in dogs, the action of  $\alpha$ -fagarine and quinidine can be considered as similar. With respect to the ventricle, however,  $\alpha$ -fagarine was the most active. Moisset de Espanés and Moyano Navarro (15) showed in dogs a depression of both conduction time and irritability following  $\alpha$ -fagarine. They reported that  $\alpha$ -fagarine may increase the cardiac frequency by removing vagal tone (atropine-like action) but with inhibition of the vagus, negative chronotropism is clearly seen. Thus, as summarized by Deulofeu (19),  $\alpha$ -fagarine produces a marked depression in all the fundamental properties of the heart.

In adequate dosage, any substance which lengthens the refractory period of heart muscle (or of nerve) can be expected to have a fibrillation-inhibiting action and to cause a decrease in the maximum frequency which the isolated guinea pig auricle can follow. Therefore, one should not expect the preparation to be a highly specific index of "quinidine-like" activity, but rather a measure of depressive action on the heart tissue resulting, in part, in a lengthening of the effective refractory period.

Moisset de Espanés (20) reported that a given concentration of  $\alpha$ -fagarine may produce an elevation in tonus, a relaxation, or a mixed response in guinea pig

pounds against  $10^{-6}$  molal histamine,  $\alpha$ -fagarine reduced the response by about 30 per cent, while papaverine caused an 80 per cent reduction, and the N-methyl derivative (II) completely suppressed the response. Better quantitative valuation was obtained by using  $10^{-5}$  molal concentrations of the compounds against  $10^{-6}$  molal histamine, and the following average reductions in response were obtained: quinidine 21 per cent, papaverine 23 per cent,  $\alpha$ -fagarine 13 per cent, metamethoxybenzyl homomyristicylamine 42 per cent, (II) 60 per cent, (III) 32 per cent, and (IV) 34 per cent.

*Acute Toxicities.* To complete the comparisons of the relative activities of  $\alpha$ -fagarine and the N-methyl metamethoxybenzyl homomyristicylamine (II) example of the provisional structure of  $\alpha$ -fagarine proposed by Deulofeu and co-workers (5, 6), a study of the respective toxicities was carried out. White mice of the same strain and weighing approximately 20 grams were used, and the appropriate quantity of a  $10^{-2}$  molal aqueous solution of the hydrochloride was injected intraperitoneally. Groups of 10 mice for each of three dosages were used

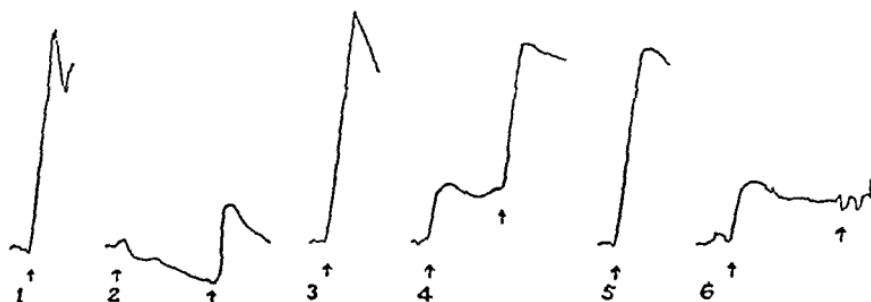


FIG. 3. Isolated guinea pig ileum tracings, with dosages given as molal concentrations: 1,  $10^{-6}$  Histamine; 2,  $10^{-4}$  Papaverine HCl followed by  $10^{-6}$  Histamine; 3,  $10^{-6}$  Histamine; 4,  $10^{-4}$   $\alpha$ -Fagarine HCl followed by  $10^{-6}$  Histamine; 5,  $10^{-6}$  Histamine; 6,  $10^{-4}$  N-Methyl-(m-Methoxybenzyl)-Homomyristicylamine HCl followed by  $10^{-6}$  Histamine.

and the  $LD_{50}$  computed from the results by the method of Bliss (11). The  $LD_{50}$  for  $\alpha$ -fagarine was found to be  $0.412 \pm 0.023$  millimols/kgm. (167 mgm./kgm. of its hydrochloride), a value which does not agree very well with the 93–100 mgm./kgm. reported by Moisset de Espanés (12). For the N-methyl derivative (II) the  $LD_{50}$  was found to be  $0.326 \pm 0.041$  millimols/kgm. (118 mgm./kgm. of its hydrochloride). In comparison with these compounds, the  $LD_{50}$  of papaverine was found to be  $0.348 \pm 0.017$  millimols/kgm. (130 mgm./kgm. of its hydrochloride).

All three of these compounds exhibit some degree of convulsant activity, but there were marked differences in the character and severity of the convulsions. Although the animals receiving 0.6 millimols/kgm. of  $\alpha$ -fagarine showed some evidences of depression they also exhibited clonic convulsions, but the respiration did not appear to be arrested by the muscular spasms. Those receiving 0.4 millimols/kgm. of (II) showed violent clonic convulsions with severe tonic extensor spasm which locked the respiratory muscles in a manner suggestive of strychnine. Following 0.4 millimols/kgm. of papaverine, the animals showed

## SUMMARY

1. The cardiac inhibitory effects of metamethoxybenzyl homomyristicylamine, its N-methyl derivative, and of 2-(metamethoxybenzyl)-6,7-methylenedioxy-8-methoxy- and 2-(metamethoxybenzyl)-6-methoxy-7,8-methylenedioxy-1,2,3,4-tetrahydroisoquinoline are similar to those of  $\alpha$ -fagarine.

2. In the lower dosage range used, the first of these compounds was comparable to  $\alpha$ -fagarine in the intensity of its inhibitory effect, while the rest of the compounds appeared to be less active. At a higher dosage range all of the compounds and  $\alpha$ -fagarine appeared to be equally inhibitory.

3. Studies on isolated ileum showed that  $\alpha$ -fagarine, unlike quinidine or papaverine, exerted a depressant effect in lower dosages and a stimulant effect at higher dosages. This stimulant effect was not abolished by atropine. The synthetic compounds also showed the stimulant as well as depressant effects, though they were quantitatively different.

4. In antagonizing the actions of acetylcholine on the ileum,  $\alpha$ -fagarine was quite inactive, while the synthetic compounds were about as active as papaverine. Against histamine,  $\alpha$ -fagarine had only limited activity, while the synthetic compounds showed considerably more activity and were more active than either quinidine or papaverine.

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intestine. Our results agree with those which he reported, but by observing the effects of a wide range of dosages on the same preparation it has become apparent that weak concentrations of  $\alpha$ -fagarine result in relaxation, that high concentrations result in contraction, and that intermediate concentrations may give transitional effects. This probably indicates the involvement of two distinct mechanisms in the response to these compounds. From the fact that transitional stages exist at dosages intermediate between those producing contraction and those which result in relaxation, it would appear that these mechanisms are simultaneously occurring and are in a greater or lesser degree of balance. The relative threshold concentrations for the two systems are, however, fairly well separated, the spasmolytic activity occurring at a much lower concentration than the stimulant action. The latter, however, is obviously the dominant action, and in adequate concentration, completely masks the relaxation of the intestine. The concentration threshold may vary considerably from preparation to preparation.

In view of the claim by Moisset de Espanés and Moyano Navarro (15) that  $\alpha$ -fagarine shows an atropine-like action in abolishing vagal tone, it is somewhat surprising to find such a slight influence on the effect of acetylcholine on the isolated ileum, inasmuch as atropine will completely suppress acetylcholine stimulation under similar conditions. It might suggest that the action of  $\alpha$ -fagarine is more intimately concerned with the muscle than with a neuro-muscular mechanism.

Probably the most significant result obtained from the present study has been the demonstration from a comparative standpoint that, although qualitatively similar,  $\alpha$ -fagarine and N-methyl metamethoxybenzyl homomyristicylamine are not identical, nor is  $\alpha$ -fagarine identical pharmacologically with any of the compounds tested. This confirms the conclusion of Redemann, Wisegarver and Icke (7) based wholly on chemical differences.\* The most striking physiologic difference in activity between  $\alpha$ -fagarine and the compound of the structure provisionally suggested by Deulofeu (5, 6) was with respect to their antagonism to histamine and acetylcholine, although the character of convulsant activity found in the toxicity studies also showed clear-cut discrepancy between the compounds.

That greater differences in activity on the isolated auricle preparation were not encountered does not argue against this concept, since, although they showed some differences at weaker concentrations, all of the compounds studied showed marked similarity in activity at higher ones. This is in line with the findings of Dawes (9, 10) that compounds of widely diverse types show "quinidine-like" activity on the isolated auricle, and indicates that the preparation lacks a sufficient specificity to clearly delineate between the compounds used in this study.

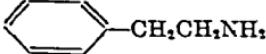
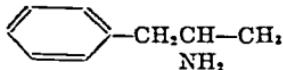
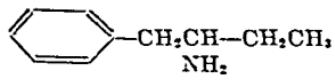
Although a relationship probably exists between the degree of depressant action of the compounds on the isolated auricle preparation and therapeutic efficacy in auricular flutter or fibrillation, the demonstration of the clinical effectiveness of these or other compounds of course must await direct clinical evaluation.

\* Since this manuscript was submitted, Surrey (J. Am. Chem. Soc. 70, 2887, 1948) reported the synthesis of N-methyl metamethoxybenzyl homomyristicylamine. He also concluded that the compound is not identical with  $\alpha$ -fagarine.

chloride per 50 cc. tissue bath was chosen as a standard concentration. After 2 minutes exposure to the drug, the bath was flushed out 3 to 5 times. The responsiveness of the segments to 10  $\mu$ gm. of epinephrine base was used as a control. Four cat hearts were prepared for perfusion by the Langendorff method and these agents were added to the perfusion fluid in amounts calculated to give concentrations of 1:10,000-1:100,000. Four guinea pig lungs were perfused by the method of Tainter, Pedden and James (5). Following constriction with 1 mgm. histamine phosphate, 1-10 mgm. of the amines were administered.

*Analeptic activity in anesthetized mice.* Groups of 10 albino mice received 65 mgm. of sodium pentobarbital (0.325 per cent solution) per kgm. intraperitoneally. One group was used as control and the other groups received 20 mgm. of phenisopropylamine hydrochloride per kgm., 20 mgm. of phenethylamine hydrochloride per kgm., or 20 mgm. of 1-phenyl-2-butylamine hydrochloride per kgm. subcutaneously, 15 minutes after the pentobarbital. The time until the mouse assumed an erect position was recorded. The number of mice that survived for 24 hours was recorded. The assay was repeated with fresh mice 4 days

TABLE 1

CHEMICAL NAME	BARBITALIZED DOGS		RABBIT JEJUNUM
Amine hydrochlorides	Pressor activity; micrograms of epinephrine equivalent to one mgm. of amine hydrochloride	Duration; time (minutes) for pressor rise to be 50% above normal	Per cent change in tone produced by 100 mgm./liter
2-Phenylethylamine	10.5 $\pm$ 2.1	1.9 $\pm$ 0.3	+100--10
			
1-Phenyl-2-propylamine	4.6 $\pm$ 1.6	8.3 $\pm$ 3.7	+50--40
			
1-Phenyl-2-butylamine	3.6 $\pm$ 1.9	14.4 $\pm$ 5.9	+10--70
			

later, and repeated again with double the amount of amines. The procedure is essentially that of Chakravarti (6).

*Oral effects in man.* Four experiments were performed in each of 3 adult males (26-34 years; 67-81 kgm.). The agents were given orally in warm water 2½ hours after a light morning or noon meal. The systolic and diastolic blood pressure and the pulse rate were recorded every 10 minutes for 3 to 4 hours while the subject remained sitting quietly. Experiments were performed once each week. One set of experiments was performed using the finger ergograph fatigue method of Alles and Feigen (7).

**RESULTS.** 1-Phenyl-2-butylamine is about 35 per cent as active a vasopressor agent as phenethylamine, and about 75 per cent as active as phenisopropylamine, on a weight basis, in barbitalized dogs. The ratios are slightly larger for 1-phenyl-2-butylamine on a molecular basis. It has been demonstrated previously (8) that phenisopropylamine is only about one-half as potent in raising the blood pressure of the barbitalized dog as phenethylamine.

# THE PHARMACOLOGICAL ACTIVITY OF 1-PHENYL-2-BUTYLAMINE

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Since the fundamental study by Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. Alles (2) showed that the introduction of a methyl group into the 1- or "alpha" position of the fundamental 2-phenylethylamine structure led to a compound, now known as amphetamine, that produced greatly prolonged sympathomimetic effects and central nervous system stimulation, even on oral administration. The next obvious step was to determine the activity of the compound with an ethyl group in place of the methyl group. This compound, 1-phenyl-2-butylamine, was one of a series of compounds investigated by Hauschild (3) in a routine survey of 27 phenylalkylamines. Since he did not find it particularly outstanding by any of the assay procedures employed, it has apparently received little further attention. In this study, 1-phenyl-2-butylamine hydrochloride<sup>1</sup> has been compared with 2-phenylethylamine hydrochloride ("beta-phenethylamine") and 1-phenyl-2-propylamine hydrochloride ("phenisopropylamine" or "amphetamine").

**EXPERIMENTAL PROCEDURE.** *Blood pressure effects in dogs.* In order to avoid the error that can be introduced by tachyphylaxis we resorted to a modification of the method of Chen (4) of standardization with an agent, such as epinephrine, that neither produces nor is affected by this phenomenon, and followed by the injection of a test dose of the agent under consideration. Eighteen apparently healthy, adult mongrel dogs (5.5-17 kgm.) were used; these were anesthetized with sodium barbital (330 mgm./kgm.) administered intraperitoneally 90 minutes prior to use. Blood pressure was recorded by the usual mercury manometer and soot kymograph. The animals were standardized with graded doses of epinephrine (1-12 micrograms/kgm.) and the 1 mgm. test dose of the agent per kgm., as a 1 per cent solution of the hydrochloride salt, was injected into a femoral vein. At varying time intervals, this dose of the same drug or a related drug was injected until the blood pressure level permanently changed, or until there was evidence of decreased responsiveness of the animal. The epinephrine equivalence data given in table I are based on the response to the first injection of a drug into the animal after the epinephrine standardization. Six animals were used for each drug. An additional 4 animals were prepared and each received 1.4 mgm. of 1-phenyl-2-butylamine hydrochloride per kgm. for comparison of the duration of action with the approximately equipressor dose (1 mgm./kgm.) of phenisopropylamine hydrochloride.

*Isolated tissue segments.* Sections of jejunum from 4 rabbits were placed in oxygenated Tyrode solution at 37-38°C. After some preliminary observations, 5 mgm. of amine hydro-

<sup>1</sup> I am grateful to Dr. Jerome Martin, Commercial Solvents Corporation, Terre Haute, Indiana, for generously supplying the phenisopropylamine and 1-phenyl-2-butylamine bases from which the corresponding hydrochlorides were prepared in this laboratory. I am grateful to D. A. Herring and F. K. Hampton for technical assistance.

Oral doses as high as 6 mgm. of phenethylamine hydrochloride per kgm. had no demonstrable effect in the individuals tested. Doses of 0.25 mgm. 1-phenyl-2-butylamine hydrochloride per kgm. produced some talkativeness but no obvious changes in blood pressure or pulse rate over a 3-hour period. The subjects stated that they felt the typical effects that occur with phenisopropylamine, such as a sense of well-being, friendliness, and the inability to stop talking. Ergograph results at this dose level of 1-phenyl-2-butylamine were not conclusive. Twice this dose of 1-phenyl-2-butylamine produced considerable blood pressure change (see fig. 2), pronounced euphoria that lasted from 12 to 16 hours, and a tremen-

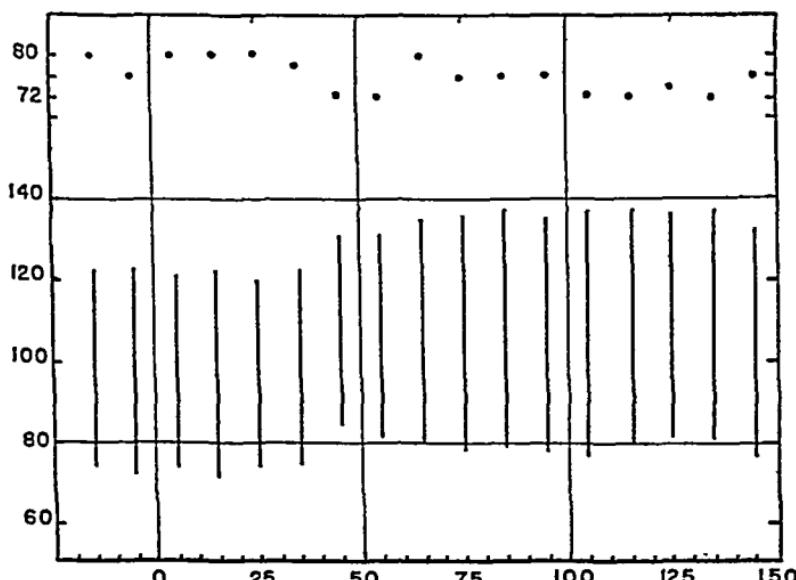


FIG. 2. Human Male (81 kgm.). Pulse rate (dots), in beats per minute, above; systolic and diastolic blood pressure (pulse pressure as solid lines), in mm. Hg, below. Time in five-minute intervals. 1-Phenyl-2-butylamine hydrochloride, 40.5 mgm., taken orally in 200 cc. water, at 0 time.

dous desire to talk and move around. The increase in ability to do work on the ergograph lasted as long as 5 hours. The following day the individuals complained of being very tired and without enthusiasm for effort of any kind. Similar blood pressure rises were produced by 0.25 mgm. of phenisopropylamine hydrochloride per kgm. and the central effects were similar although not so prominent or prolonged. The effects the following day were not so severe. Tentatively, from these few experiments, it may be concluded that 1-phenyl-2-butylamine is about one-half as active a vasopressor agent as phenisopropylamine and either more than one-half as active or longer acting as a central nervous system stimulant following oral administration in man.

**DISCUSSION.** The introduction of an ethyl group in the phenethylamine molecule produces a compound which causes a more prolonged vasopressor action than that by the compound with a methyl group in the same position. Presumably this may be due to an even greater difficulty in destruction by body enzymes

The duration of action relationships are quite different, with half the pressor rise (9) following the administration of 1 mgm. of 1-phenyl-2-butylamine lasting about twice as long as that after 1 mgm. of phenisopropylamine and seven times as long as that from phenethylamine. Doses of 1-phenyl-2-butylamine (1.4 mgm.) equipressor to 1 mgm. of phenisopropylamine have only a slightly longer duration for the half-rise (average 18.1 minutes in 4 dogs). Like other long duration vasopressor amines (9), 1-phenyl-2-butylamine evidences tachyphylaxis on repeated administration (see figure 1).

The effects of 1-phenyl-2-butylamine in isolated tissue preparations are no more definite than those of phenethylamine and phenisopropylamine. As an indication of this, the extremes of the results with these agents in the Magnus preparation have been given in table 1. The averages of the results indicate that

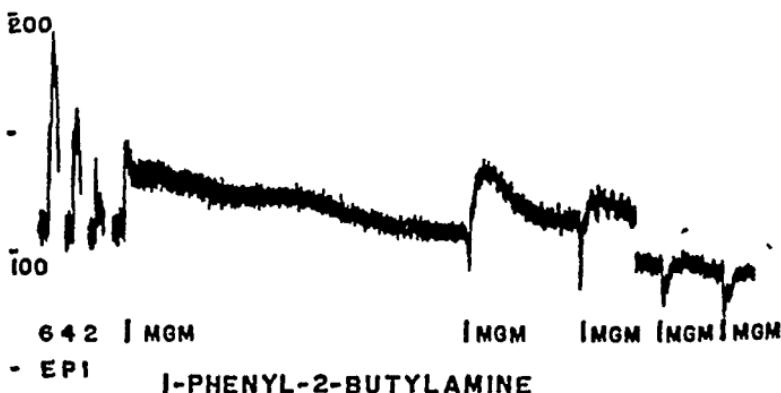


FIG. 1. Dog (17 kgm. female). 330 mgm. Na Barbital per kgm. Blood pressure in mm. Hg, ordinate; time, in 10 minute intervals, abscissa. Epinephrine given at EPI, doses in micrograms base per kgm., eight minute intervals between injections. 1-Phenyl-2-butylamine hydrochloride, 1 mgm./kgm., given at each 1 MGM. Record run continuously except for breaks between epinephrine injections and a 30-minute interval (drum stopped 20 minutes) between third and fourth injections of 1-phenyl-2-butylamine.

1-phenyl-2-butylamine is most relaxant, with phenisopropylamine next, and phenethylamine predominantly contractile in action. Similarly, all these agents produce inhibition of rate, force of contraction, and rate of coronary outflow in the perfused heart, with the 1-phenyl-2-butylamine being most depressant for the cat heart. Hauschild (3) obtained similar results for guinea pig heart. None of the 3 agents antagonized the effects of histamine in the perfused guinea pig lung.

Twenty mgm. of phenisopropylamine hydrochloride per kgm. caused the pentobarbitalized mice to awaken in  $35.4 \pm 6.3$  minutes, while 20 mgm. 1-phenyl-2-butylamine caused them to awaken in  $41.3 \pm 12.2$  minutes; the controls awakened in  $57.4 \pm 11.0$  minutes. Larger doses of these agents, and the doses of phenethylamine prolonged the sleeping time. Whether or not the mice awakened in greater or less time than the controls, more mice always died that received the analeptics, with the greatest mortality after the phenisopropylamine.

# STUDIES ON THE TOXICITY, DISTRIBUTION AND EXCRETION OF EMETINE<sup>1,2</sup>

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Although emetine is widely used in the treatment of amebic dysentery and is specific for the treatment of amebic abscess of the liver very little is known about its distribution, fate and excretion. The study presented here furnishes some basic information concerning the metabolism of the drug in the animal body.

Emetine frequently causes toxic symptoms especially noted when its action is cumulative from high dosage or from prolonged therapeutic use. Its toxic effects, especially on the heart (1), have frequently been observed in patients receiving therapeutic doses (2, 3, 4). Boyd and Scherf (5) in experiments on dogs and cats demonstrated that much more pronounced effects result from the second and third injections of an equal amount of the drug, although the normal electrocardiogram had been restored. Lake (6) and Rosen *et al.* (7) have shown that divided doses over a period of several days are almost as toxic as the total dose given at one time.

**PROCEDURES AND RESULTS. Methods.** Two methods for the determination of emetine were employed. In the first, the reaction of the drug with methyl orange (8) was utilized. The reagents were prepared as previously described (8). Standard curves were obtained by adding known amounts of emetine to aqueous suspensions of homogenized rat and dog tissues and determining in duplicate the emetine content by a modification of Brodie and Udenfriend's method. By using a 0.1 M phosphate buffer of pH 7.5 to wash the alkaline ethylene dichloride extract of tissue, it was possible to decrease blank readings to an optical density of 0.02 or less. Inasmuch as this buffer wash does not decrease emetine readings, greater specificity in the method was obtained in addition to the increased accuracy at low values. Since emetine was found to be firmly bound to the tissue proteins, a 30 per cent trichloroacetic acid solution was used to precipitate the proteins before extracting the emetine into the ethylene dichloride.

For tissue determination, weighed samples were added to 15 or 20 cc. of distilled water and homogenized in a Waring blender. Five cc. of 30 per cent trichloroacetic acid were added to 5 cc. of the homogenate and the mixture shaken. Five cc. of 5 N NaOH and 10 cc. of ethylene dichloride were added and shaken for five minutes. The flask contents were transferred to a Pyrex ignition tube, centrifuged for five minutes and the supernatant aqueous layer removed by aspiration. The ethylene dichloride layer was then placed in a flask containing 5 cc. of 0.1 M phosphate buffer. The contents were thoroughly mixed by shaking for five minutes, transferred to an ignition tube and centrifuged five minutes. The supernatant layer was completely removed by aspiration and the ethylene dichloride decanted

<sup>1</sup> A preliminary report was presented at a meeting of the American Society for Pharmacology and Experimental Therapeutics, Federation Proc. 7, 256 (1948).

<sup>2</sup> Aided by a grant from the National Cancer Institute, National Institutes of Health.

<sup>3</sup> Part of this data was used in a thesis presented by Mr. Gimble to Columbian College George Washington University for the degree of Master of Science.

than is the case with phenisopropylamine as compared to phenethylamine. The addition of the ethyl group produces a greater interference with the action of the compound when given intravenously in dogs than the methyl group; both compounds are inferior in action to the simple phenethylamine. However, either an ethyl or methyl group protects the compound from destruction so that it is active orally. The central nervous system, which requires the phenylisopropylamine grouping as a minimum for an agent to be active as a stimulant, will also respond to the 1-phenyl-2-butylamine.

The three agents are almost non-specific in their action on isolated smooth muscle and similar preparations and in this respect are like almost all the other agents that do not have a hydroxyl group on the ring or in the side-chain (8-10).

#### SUMMARY

1-Phenyl-2-butylamine hydrochloride is about 1/275 as potent a vasopressor agent as epinephrine, and about 35 per cent as active as beta-phenethylamine and about 75 per cent as active as phenisopropylamine, or amphetamine, in barbitalized dogs. In 1 mgm./kgm. doses, 1-phenyl-2-butylamine has a duration of pressor effect that is about twice that of amphetamine, and seven times that of phenethylamine.

1-Phenyl-2-butylamine is most relaxant for isolated rabbit jejunum in concentrations of 100 mgm./l., phenisopropylamine next, and phenethylamine least relaxant, and often contractile in action. All three agents depress the function of the isolated perfused cat heart, and are ineffective against histamine spasm in perfused guinea pig lungs.

Twenty mgm. of phenisopropylamine hydrochloride per kgm. shortens the sleeping time of pentobarbitalized mice the most, with 1-phenyl-2-butylamine next, and phenethylamine inactive.

Oral doses as high as 6 mgm. of phenethylamine hydrochloride per kgm. had no demonstrable effect in man. As little as 0.25 mgm. of 1-phenyl-2-butylamine hydrochloride had some central nervous system stimulant effect, and 0.5 mgm./kgm. produced pronounced central nervous stimulant effects concomitant with a prolonged rise in blood pressure. One-fourth mgm. phenisopropylamine hydrochloride per kgm. produced a similar rise in blood pressure and central nervous system effects, although not so prolonged.

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the Waring blender, and portions of the tissue suspension were assayed for emetine by the methyl orange technique. Another portion of the tissue suspensions was analyzed by the ultraviolet spectrophotometric technique. Table 1 shows the comparison. These results indicate that the values obtained for emetine concentrations using the modified methyl orange technique are concentration of true emetine.

*Acute toxicity.* The acute toxicity of emetine was studied in rats and mice. The animals were injected intraperitoneally with varying doses of emetine hydrochloride and were observed over a seven-day period. Using fifty mice the LD<sub>50</sub> was calculated and found to be  $62 \pm 2.3$  mgm. per kgm. Thirty rats were used in this study and the LD<sub>50</sub> was found to be  $17.2 \pm 1.4$  mgm. per kgm. The data are presented in table 2.

TABLE 1  
*Comparison of colorimetric and ultraviolet absorption methods*

SPECIMEN	COLORIMETRIC	ULTRAVIOLET ABSORPTION	PER CENT DIFFERENCE
	mgm.	mgm.	
(1)	.082	.075	9
(2)	.089	.096	8
(3)	.076	.080	5

TABLE 2  
*Toxicity of emetine hydrochloride in rats and mice*

mgm. per kgm. ....	50	60	70	80	100
no. of deaths ..	1	9	6	10	8
no. of mice .	6	14	10	12	8
mgm. per kgm.	10		20		25
no. of deaths .	1		3		10
no. of rats	10		10		10

*Rate of destruction of emetine.* Emetine hydrochloride was injected intraperitoneally in single doses of 40 mgm. per kgm. into several groups of 8 mice each and the emetine content of entire animals was determined at intervals up to thirty-five days.

Each animal was homogenized in distilled water in the Waring blender, using two parts of water to one part of tissue. A 5 cc. aliquot of the tissue suspension was analyzed for emetine content according to the methyl orange method. A series of control animals was treated as above and the blank values obtained were subtracted from the values obtained in the emetine injected animals.

Emetine levels in the animal body decreased slowly (fig. 2) until the eighth day when approximately 35 per cent was still in the animal body. Another group of animals assayed for emetine content thirty-five days after injection had also retained approximately 35 per cent of the drug indicating extensive storage of emetine and very slow disposition of the drug after the first week.

into shaking flasks containing 0.5 cc. of methyl orange. After shaking for three minutes, the excess methyl orange was carefully removed by centrifuging and aspiration. After recentrifuging the ethylene dichloride, 5 cc. were pipetted into a colorimeter tube containing 1 cc. of alcoholic  $H_2SO_4$ . The color developed was read in the Coleman Junior spectrophotometer at 540  $\text{m}\mu$  with distilled water being used to set the instrument at zero optical density.

In the second method, standard curves were prepared by adding known amounts of emetine to aqueous suspensions of homogenized rat and dog tissues and the emetine was determined by the ultraviolet absorption spectra in the Beckman spectrophotometer. The tissues were prepared in the same way as for the methyl orange method except that the ethylene dichloride extract was washed twice with pH 8.0, 0.02 M borate buffer instead of

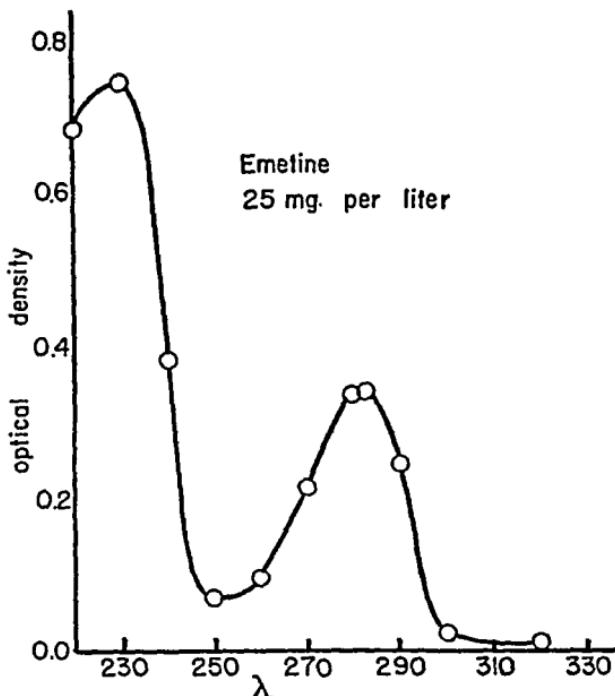


FIG. 1. ULTRAVIOLET LIGHT ABSORPTION OF EMETINE DISSOLVED IN 0.1 M SULFURIC ACID

with phosphate buffer. After the final centrifugation the supernatant layer was completely removed by aspiration and the ethylene dichloride decanted into a shaking flask containing 4 cc. of 0.1 N  $H_2SO_4$ . After shaking for five minutes, the aqueous layer was siphoned off and transferred to a quartz tube. The absorption bands of the emetine were then read in the Beckman spectrophotometer (fig. 1). This was used as the standard reference curve for the determination of emetine concentration in tissues.

Since the methyl orange technique is not specific for emetine, the values obtained in the experiments using this method would indicate apparent emetine rather than true emetine. To ascertain the specificity of the modified methyl orange method, ultraviolet absorption bands were read on extracts of livers from rats injected intraperitoneally with emetine. Three rats were injected intraperitoneally with emetine (10 mgm. per kgm.) and the animals sacrificed after two hours. The livers were then homogenized with distilled water in

*Distribution in dog tissue.* A dog was injected with emetine hydrochloride, 1 mgm. per kgm. on six successive days and the tissues analyzed twenty-four hours after the last dose. The distribution of emetine in the dog follows closely that of the rat. The results in mgm. of emetine per kgm. of fresh tissue were as follows: liver, 31; kidney, 17; spleen, 15; lung, 11; heart, 2; muscle, 2. Here the cumulative action of emetine was again indicated.

*Excretion.* Studies of the excretion of emetine were carried out in rats, dogs and in one human. The rats were injected intraperitoneally with a 2 mgm. per kgm. dose of emetine; the urines were collected in periods of one to five days after

TABLE 3  
*Distribution of emetine in rat tissues*

TIME AFTER DRUG	MG.M. PER KGM. FRESH TISSUE							
	blood	brain	heart	kidney	liver	lung	muscle	spleen
2 hrs.	0.0	5.6	2.0	19.4	28.5	14.0	1.2	25.0
	0.2	2.5	4.8	5.7	10.5	6.2	1.1	11.1
	0.0	2.9	5.0	15.8	26.0	5.4	1.5	5.4
	4.8	17.3	3.6	34.8	37.0	30.6	2.0	46.5
	—	3.8	6.2	14.5	55.0	25.8	1.1	21.8
Mean...	1.2	6.4	4.3	18.0	31.4	16.4	1.4	22.0
24 hrs.	0.0	3.0	0.0	17.7	23.0	16.6	1.2	29.2
	0.7	5.9	2.8	11.2	20.0	17.2	1.7	26.3
	0.0	7.7	0.0	8.6	19.0	6.2	1.4	20.0
	1.8	6.0	10.5	14.3	28.5	17.0	5.1	24.4
	9.4	5.1	7.7	15.7	29.8	12.7	1.8	24.5
Mean...	2.2	5.5	4.4	13.5	24.1	13.9	2.2	24.9
48 hrs.	1.3	3.5	6.4	12.4	12.8	12.7	1.4	29.3
	0.0	4.0	4.2	10.8	10.2	8.1	1.5	38.1
	1.2	18.2	8.1	18.5	23.3	21.0	2.2	—
	1.3	3.2	5.6	14.1	15.5	8.4	1.3	10.5
	3.0	2.3	0.0	7.9	15.9	6.2	1.1	11.9
Mean...	1.4	6.2	4.9	12.7	14.5	11.2	1.5	22.4

injection and analyzed for emetine according to the methyl orange method. Since the urine blanks in the methyl orange technique were rather high, this method was not considered to be accurate or specific for the determination of emetine in urine. Nevertheless the results showed no appreciable amount of apparent emetine to be present. When absorption bands were read on extracts of these urine samples, no peak was obtained at 282 m $\mu$ , and the shape of the curves did not correspond to the standard emetine absorption curve determined earlier with a known amount of emetine.

Twenty-four hour urine specimens were collected from three dogs injected

In another group the dose of emetine was increased to 80 mgm. per kgm. and it was found that the emetine content was approximately the same absolute level after eight days as in the group receiving 40 mgm. per kgm. This indicates that emetine is appreciably stored in the animal body and suggests that the animal will store only a certain amount of the drug, above which amount the emetine is either destroyed or excreted.

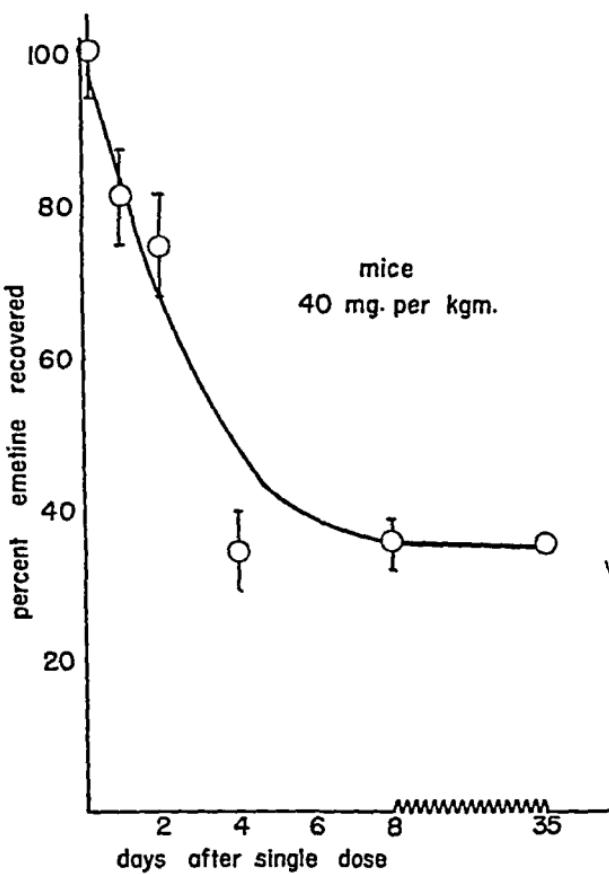


FIG. 2. RATE OF DISPOSITION OF EMETINE IN MICE AFTER INTRAPERITONEAL INJECTION

*Distribution in rat tissue.* In three series of 5 rats each, emetine hydrochloride (10 mgm. per kgm.) was injected intraperitoneally and tissues analyzed by the methyl orange method two, twenty-four and forty-eight hours after injection. After correcting for the tissue blanks, the concentration of emetine present was obtained by reference to the standard curve. The data are presented in table 3.

The results show that most of the emetine is stored in the tissues. By far the highest concentration was in the liver, with lower concentrations in kidney, spleen and lung, and very little in the brain, heart, muscle and blood.

the number of the bottle. As a control a known amount of emetine was added to a sample of blank urine and run through the distribution procedure described above. The distribution curves obtained did not indicate any appreciable amount of emetine in any sample. The results of one distribution are illustrated in fig. 3. This was from the urine giving the highest amount of methyl orange reactants.

Through the kindness of Major T. A. Haedicke of Walter Reed Hospital the urine samples were obtained from one patient during a course of emetine administration. A total of 600 mgm. of the hydrochloride was given over a period of 10 days and urine samples were collected for each twenty-four hours. These were each made alkaline and extracted with ether. The extract was evaporated at

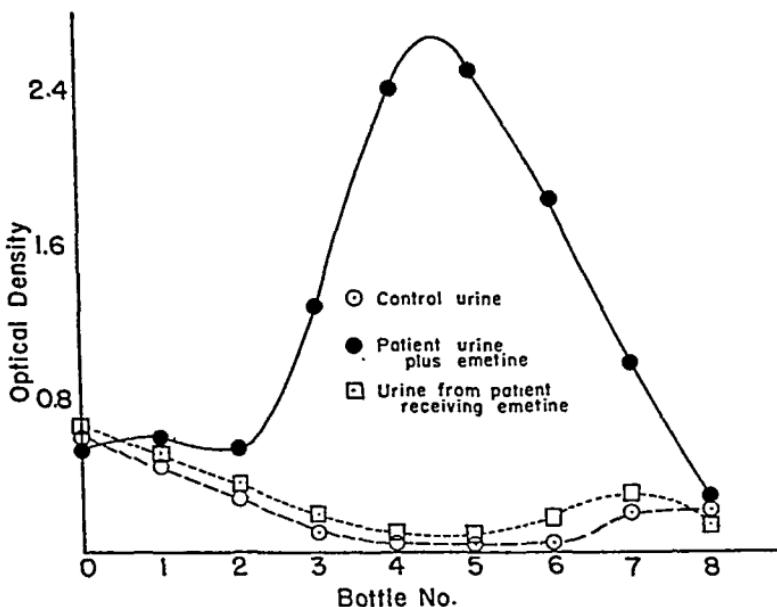


FIG. 4. COMPARISON OF 8 PLATE COUNTER-CURRENT DISTRIBUTIONS OF THE URINE FROM A PATIENT RECEIVING EMETINE WITH THAT OF A CONTROL URINE AND WITH THE SAME URINE FROM THE PATIENT TO WHICH 2 MGM. EMETINE HYDROCHLORIDE HAD BEEN ADDED

low temperature and dissolved in 50 cc. of ethylene dichloride and half of it used for an eight plate counter-current distribution. In no case was there evidence of an appreciable amount of emetine. The results on the urine for the seventh day are shown in fig. 4. Similar results were obtained with the other samples. The results indicate that the amount of emetine excreted in any twenty-four hour period was either negligible or no more than a few milligrams.

DISCUSSION. Rosen *et al.* (2) found that the toxicity of emetine administered subcutaneously to guinea pigs is approximately the same whether the alkaloid is given in a single large dose or in repeated doses of  $\frac{1}{2}$  the acute MLD over a period of forty days. Lake (1) obtained similar results in rats. Our results of the experiments on the tissue distribution of emetine in mice, rats, and dogs are in agreement with the above observations. Thirty-five days after a single injec-

with 1 mgm. per kgm. of emetine daily. Dog No. 1 received a total dose of 90 mgm. of emetine over a seven-day period. Dog No. 2 received a total dose of 203 mgm. of emetine over a thirteen-day period. Dog No. 3 received a total dose of 84 mgm. of emetine over an eight-day period. Several urine samples were examined for emetine by the ultraviolet absorption method, but there was no detectable amount of the drug present.

In addition, attempts to fractionate and characterize the urinary products were carried out on an extract of the total amount of urine collected from each dog by a modification of Craig's counter-current technique (9). The urine was

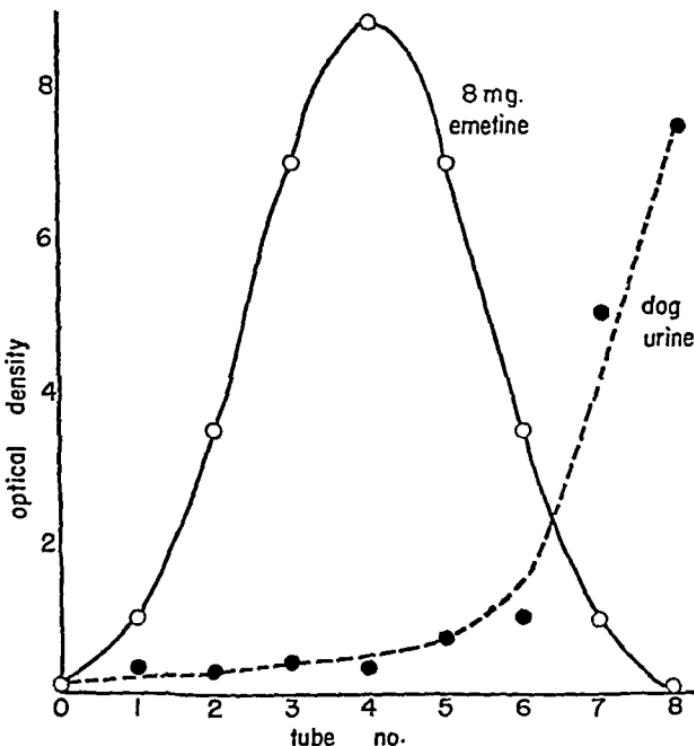


FIG. 3. COMPARISON OF 8 PLATE COUNTER-CURRENT DISTRIBUTIONS OF THE URINE OF A DOG RECEIVING EMETINE WITH THAT OF A SOLUTION OF PURE EMETINE

acidified with  $H_2SO_4$  and then extracted with ether to remove interfering substances that would cause emulsion formation. The resulting urine was then made alkaline with concentrated NaOH and reextracted with ether. This was evaporated at a low temperature and dissolved in 50 cc. of ethylene dichloride. Half of this was then carried through an eight transfer separation in a system consisting of equal parts of ethylene dichloride (25 cc.) and 2 M phosphate buffer pH 5.5, allowing the lower solvent phase to migrate. The ethylene dichloride and phosphate buffer layers were then analyzed for emetine by the usual methyl orange method. The characteristic distribution curves were then obtained by plotting the optical density of the methyl orange reactants in each bottle against

# EXPERIMENTAL HEART FAILURE CAUSED BY CENTRAL NERVOUS SYSTEM DEPRESSANTS AND LOCAL ANESTHETICS<sup>1</sup>

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Drugs possessing a negative inotropic cardiac action have proven to be a useful tool for the analysis of the changes associated with impaired myocardial contractility and for the study of substances which increase the contractile power of the heart (1). In a series of investigations in this laboratory on the mechanism of experimental myocardial failure of the mammalian heart, a number of such drugs, chosen from among the local anesthetics and central nervous system depressants, were made use of. Some of these drugs had been known from the literature to depress the contractility of the mammalian heart. Examples are cocaine (2), barbiturates (3), diphenylhydantoin (4), and paraldehyde (5, 6). Others, such as the more recently introduced oxazolidinedione derivatives, have never been examined before for their effects on the heart. The present paper is an account of the toxic action of these and other local anesthetics and central nervous system depressants on the heart of the dog as observed in the heart-lung preparation. The experiments were standardized in such a way as to make the results comparable with respect to the negative inotropic effect.

**METHODS.** Starling heart-lung preparations (HLP's) of dogs weighing between 8 and 12 kilograms were used. The preparations were set up and their activity was measured as specified in a previous communication (7). The ability of the heart to handle an increase in blood supply was selected as the principal criterion for estimating cardiac competence. It was determined according to a method described previously (1) from the response of the right atrial pressure to a rise of the blood level in the venous inflow vessel. The results of these determinations have been expressed as the "competence index" of the heart (7), which is the ratio  $\frac{\text{increase in inflow level} - \text{increase in right atrial pressure}}{\text{increase in inflow level}}$ . The increase in the inflow level was always 50 mm.

The compounds studied were: the central nervous system depressants paraldehyde, chlorobutanol, pentobarbital sodium, propazone (5,5-di-n-propyloxazolidinedione-2,4) sodium<sup>2</sup>, trimethadione<sup>3</sup> (3,5,5-trimethyloxazolidinedione-2,4), and diphenylhydantoin sodium; and the local anesthetics, cocaine hydrochloride, procaine hydrochloride, and tetracaine hydrochloride. Three to six experiments were performed with each drug. The drugs were injected in repeated doses into the tube leading to the venous inflow vessel. Administration was begun shortly after the heart-lung preparations were set up and was continued until a fairly severe heart failure had developed. This required usually not more than one hour. At this time the heart in the unpoisoned heart-lung preparation is still performing well.

The concentrations of drug have been given as fractions of the weights of the heart-lung

<sup>1</sup> This work was supported by a grant from the Life Insurance Medical Research Fund.

<sup>2</sup> Made from propazone kindly supplied by Dr. M. A. Thorpe of Mallinckrodt Chemical Works.

<sup>3</sup> Kindly supplied by Dr. R. K. Richards of Abbott Laboratories.

tion of emetine approximately one-third of the drug was still present in the mouse. In addition high emetine concentrations were obtained in the tissues of rats and dogs twenty-four to forty-eight hours after sub-lethal doses of the drug. This indicates a very slow disposition of the drug with a resulting high toxicity of the drug after repeated small doses.

An interesting finding in the tissue distribution results was a low concentration of emetine in the heart, despite the fact that one of the main toxic effects of emetine is on the heart. Dack and Moloshok (10) observed that after a course of emetine therapy the electrocardiographic abnormalities, when present, were of long duration, suggesting to them prolonged fixation of the drug in the myocardium. The results obtained, both in rats and dogs, indicate that the heart may be hypersensitive to emetine, since the concentrations in heart muscle were not significantly higher than those in skeletal muscle and appreciably lower than in most other organs. The hearts of two dogs given emetine were congested in appearance at autopsy with generalized marked dilation of the right auricles and ventricles. In contrast, the spleen, which contained a fairly high emetine concentration, appeared quite normal. The lungs, which also contained appreciable amounts of emetine, appeared normal except for a slight amount of pulmonary edema in one of the animals. This condition was probably caused by the failure of the right auricle and ventricle.

It is of interest to note that the liver contains the highest concentration of emetine in comparison with the other organs. This high concentration may account for the effectiveness of the drug in eradicating amebic abscesses.

#### SUMMARY

1. The LD<sub>50</sub> of emetine in mice was 62 mgm. per kgm. and in rats, 17 mgm. per kgm.
2. The relative concentrations of emetine in rat and dog tissues were similar and were of the following order: liver > kidney > spleen > lung > brain > heart > skeletal muscle > blood.
3. Emetine was stored in the tissues of mice, rats, and dogs and was very slowly destroyed or excreted.
4. Emetine was not excreted in the urine to any appreciable extent.

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Drugs possessing a negative inotropic cardiac action have proven to be a useful tool for the analysis of the changes associated with impaired myocardial contractility and for the study of substances which increase the contractile power of the heart (1). In a series of investigations in this laboratory on the mechanism of experimental myocardial failure of the mammalian heart, a number of such drugs, chosen from among the local anesthetics and central nervous system depressants, were made use of. Some of these drugs had been known from the literature to depress the contractility of the mammalian heart. Examples are cocaine (2), barbiturates (3), diphenylhydantoin (4), and paraldehyde (5, 6). Others, such as the more recently introduced oxazolidinedione derivatives, have never been examined before for their effects on the heart. The present paper is an account of the toxic action of these and other local anesthetics and central nervous system depressants on the heart of the dog as observed in the heart-lung preparation. The experiments were standardized in such a way as to make the results comparable with respect to the negative inotropic effect.

**METHODS.** Starling heart-lung preparations (HLP's) of dogs weighing between 8 and 12 kilograms were used. The preparations were set up and their activity was measured as specified in a previous communication (7). The ability of the heart to handle an increase in blood supply was selected as the principal criterion for estimating cardiac competence. It was determined according to a method described previously (1) from the response of the right atrial pressure to a rise of the blood level in the venous inflow vessel. The results of these determinations have been expressed as the "competence index" of the heart (7), which is the ratio  $\frac{\text{increase in inflow level} - \text{increase in right atrial pressure}}{\text{increase in inflow level}}$ . The increase in the inflow level was always 50 mm.

The compounds studied were: the central nervous system depressants paraldehyde, chlorobutanol, pentobarbital sodium, propazone (5,5-di-n-propyloxazolidinedione-2,4) sodium<sup>2</sup>, trimethadione<sup>3</sup> (3,5,5-trimethyloxazolidinedione-2,4), and diphenylhydantoin sodium; and the local anesthetics, cocaine hydrochloride, procaine hydrochloride, and tetracaine hydrochloride. Three to six experiments were performed with each drug. The drugs were injected in repeated doses into the tube leading to the venous inflow vessel. Administration was begun shortly after the heart-lung preparations were set up and was continued until a fairly severe heart failure had developed. This required usually not more than one hour. At this time the heart in the unpoisoned heart-lung preparation is still performing well.

The concentrations of drug have been given as fractions of the weights of the heart-lung

<sup>1</sup> This work was supported by a grant from the Life Insurance Medical Research Fund.

<sup>2</sup> Made from propazone kindly supplied by Dr. M. A. Thorpe of Mallinckrodt Chemical Works.

<sup>3</sup> Kindly supplied by Dr. R. K. Richards of Abbott Laboratories.

tion of emetine approximately one-third of the drug was still present in the mouse. In addition high emetine concentrations were obtained in the tissues of rats and dogs twenty-four to forty-eight hours after sub-lethal doses of the drug. This indicates a very slow disposition of the drug with a resulting high toxicity of the drug after repeated small doses.

An interesting finding in the tissue distribution results was a low concentration of emetine in the heart, despite the fact that one of the main toxic effects of emetine is on the heart. Dack and Moloshok (10) observed that after a course of emetine therapy the electrocardiographic abnormalities, when present, were of long duration, suggesting to them prolonged fixation of the drug in the myocardium. The results obtained, both in rats and dogs, indicate that the heart may be hypersensitive to emetine, since the concentrations in heart muscle were not significantly higher than those in skeletal muscle and appreciably lower than in most other organs. The hearts of two dogs given emetine were congested in appearance at autopsy with generalized marked dilation of the right auricles and ventricles. In contrast, the spleen, which contained a fairly high emetine concentration, appeared quite normal. The lungs, which also contained appreciable amounts of emetine, appeared normal except for a slight amount of pulmonary edema in one of the animals. This condition was probably caused by the failure of the right auricle and ventricle.

It is of interest to note that the liver contains the highest concentration of emetine in comparison with the other organs. This high concentration may account for the effectiveness of the drug in eradicating amebic abscesses.

#### SUMMARY

1. The LD<sub>50</sub> of emetine in mice was 62 mgm. per kgm. and in rats, 17 mgm. per kgm.
2. The relative concentrations of emetine in rat and dog tissues were similar and were of the following order: liver > kidney > spleen > lung > brain > heart > skeletal muscle > blood.
3. Emetine was stored in the tissues of mice, rats, and dogs and was very slowly destroyed or excreted.
4. Emetine was not excreted in the urine to any appreciable extent.

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magnitude may occur spontaneously in the heart-lung preparation within the time limits of the present experiments, the observed changes are probably not significant. Sharp and undoubtedly specific increases in coronary flow are pro-

TABLE 1

*Action of various central nervous system depressants and local anesthetics on the heart-lung preparation of the dog*

AGENT	NUMBER OF EXPTS.	CONCENTRA-TION PER KGM. HLP <i>mgm.</i> <i>milli-moles</i>	VENTRICULAR RATE <i>min.<sup>-1</sup></i>	MEAN SYSTEMIC ARTERIAL PRESSURE			RIGHT ATRIAL PRESSURE <i>mm. H<sub>2</sub>O</i>	SYSTEMIC OUTPUT <i>cc./ min.</i>	CORONARY OUTPUT <i>cc./ min.</i>	TOTAL OUTPUT <i>cc./ min.</i>	WORK <i>kgm.- r./ min.</i>	COMPARISON INDEX <sup>1</sup>
				PULMONARY ARTERIAL PRESSURE <i>mm. Hg</i>	MEAN PULMONARY ARTERIAL PRESSURE <i>mm. Hg</i>	RIGHT ATRIAL PRESSURE <i>mm. Hg</i>						
Diphenylhydantoin sodium	5	0 0	164	108	14	27	488	66	554	.87	.82	
		90 0.33	112	60	13	98	28	138	166	.20	.10	
Pentobarbital sodium	6	0 0	156	101	15 <sup>1</sup>	24	436	74 <sup>1</sup>	510 <sup>1</sup>	.80 <sup>1</sup>	.91	
		119 0.48	141	94	16 <sup>1</sup>	77	166	149 <sup>1</sup>	315 <sup>1</sup>	.39 <sup>1</sup>	.15	
Chlorobutanol	4	0 0	160	119	14	25	410	82 <sup>1</sup>	492 <sup>1</sup>	.88 <sup>1</sup>	.83	
		196 1.10	131	107	20	66	116	281 <sup>1</sup>	397 <sup>1</sup>	.63 <sup>1</sup>	.28	
Propazone sodium	4	0 0	137	96	15	46	348	57	405	.61	.78	
		2080 10.0	113	87	16	86	120	65	185	.26	.11	
Trimethadione	3	0 0	135	98	12	18	468	45	513	.76	.89	
		2372 16.6	135 <sup>2</sup>	79	13	73	177	76	253	.31	.14	
Paraldehyde	5	0 0	160	107	16	32	465	55	520	.86	.82	
		2378 18.0	139	92	16	84	137	147	284	.41	.12	
Cocaine hydrochloride <sup>3</sup>	5	0 0	149	94	18 <sup>1</sup>	25	608	45 <sup>1</sup>	653 <sup>1</sup>	.99 <sup>1</sup>	.89	
		42 0.12	123 <sup>4</sup>	83	28 <sup>1</sup>	61	344	37 <sup>1</sup>	381 <sup>1</sup>	.57 <sup>1</sup>	.31	
Tetracaine hydrochloride	3	0 0	156	98	11	23	378	66	444	.65	.89	
		45 0.15	99 <sup>4</sup>	86	19	81	156	82	238	.33	.15	
Procaine hydrochloride	4	0 0	141	99	17	20	445	48	493	.77	.82	
		282 1.04	111 <sup>4</sup>	90	21	77	199	84	283	.42	.15	

<sup>1</sup> 3 Experiments.

<sup>2</sup> Heart rate is increased by smaller doses.

<sup>3</sup> Experiments performed in collaboration with Dr. E. Moisset de Espanés.

<sup>4</sup> Irregular beat.

<sup>5</sup> For explanation see text.

duced by pentobarbital, diphenylhydantoin, paraldehyde, and chlorobutanol. Particularly the last-named drug is a powerful coronary dilator, as was recognized a number of years ago (12, 13). In the chlorobutanol failures in table 1 the coro-

preparations, i.e. of the combined weights of the heart, lungs, and blood. The weight of the heart was determined after each experiment, that of the lungs was taken to be 1/75 of the body weight (8). The weight of the blood was about 800 grams. For the comparison of their potency of action the drugs have been assumed to be evenly distributed in the heart-lung system.

**RESULTS.** The results are summarized in table 1. The figures given are the means of two sets of measurements, one taken before the administration of the drugs and the other taken after administration was completed.

*Negative Inotropic Action.* The severity of heart failure was fairly uniform in all experiments. The competence index decreased from initial values ranging between 0.8 and 0.9 to values of 0.1 to 0.3 (table 1). The decrease in cardiac competence is also reflected in pronounced decreases in systemic output, systemic arterial pressure, and work, and in a sharp increase in right atrial pressure. Table 1 shows that cocaine and tetracaine have a strong negative inotropic action. The concentration of these compounds required to produce a fall of the competence index from 0.90 to 0.20 is found by extrapolation or interpolation of the observed values to be 0.14 millimoles per kgm. HLP. Diphenylhydantoin and pentobarbital are, respectively, 1/2 and 1/3 as potent as the two local anesthetics. Next in order of potency follow procaine and chlorobutanol, and finally propazone, trimethadione, and paraldehyde. These last three compounds have a very weak negative inotropic action on the heart. The calcium salt of propazone was found to have a slight positive inotropic action, due to the preponderance of the action of calcium over that of propazone.

*Chronotropic Action.* All drugs tested, with the exception of trimethadione, decrease the rate of the isolated heart (table 1). This action usually parallels the decrease in contractility. In the case of cocaine and tetracaine, it may precede it. Cocaine, tetracaine, and procaine also cause irregularities of rhythm. This effect of local anesthetics is well-known (9, 10). Diphasic chronotropic effects of cocaine, which were reported in the earlier literature but denied by Kuroda (2), were not observed in the present experiments, although care was taken to administer this compound slowly in graded doses. The only drug in table 1 found capable of increasing the heart rate is trimethadione. The maximal increase is about 14 beats per minute and is obtained at a concentration of about 10 millimoles per kgm. HLP. In contrast, the other anticonvulsant studied, diphenylhydantoin, has a pronounced negative chronotropic effect (cf. also 4). The electrocardiogram recorded in diphenylhydantoin failure shows flattening of the P wave and prolongation of the P-R interval. Similar observations were made by Scherf (11) following intravenous administration of diphenylhydantoin to dogs.

*Action on Coronary Blood Flow.* The values given in table 1 for the coronary blood flow represent 10/6 of the outflow from the coronary sinus. The latter was collected with a Morawitz cannula. Table 1 shows that there is a slight decrease in coronary blood flow following the administration of cocaine. Coronary blood flow is increased by 8 to 36 cc. per minute in heart failure induced by propazone, tetracaine, trimethadione, and procaine. Since increases in coronary flow of this

sants, pentobarbital, chlorobutanol, paraldehyde, propazone, diphenylhydantoin, and trimethadione; and the local anesthetics, cocaine, procaine, and tetracaine. A comparison has been made of the intensity of the negative inotropic cardiac action of these compounds, and other phases of their toxic action on the heart have been described.

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nary output was more than twice as large as the systemic output, averaging almost 300 cc. per minute.

*Action on Pulmonary Circulation.* The pulmonary arterial pressure is markedly increased in heart failure caused by the local anesthetics and by chlorobutanol (table 1). It remains practically unchanged in heart failure induced by paraldehyde, pentobarbital, diphenylhydantoin, propazone, and trimethadione.

**DISCUSSION.** The drug concentrations in table 1 exceed by far those in the therapeutic range. In the case of the local anesthetics they are also considerably higher than the minimal lethal concentrations for the dog and other mammals after intravenous administration (see 9). This applies even to cocaine, which is as toxic to the myocardium as tetracaine, although its lethal toxicity is only half as great. Procaine, which is a much weaker local anesthetic and systemic poison than tetracaine and cocaine, has a correspondingly weaker action on the heart. The concentrations of the central nervous system depressants used to produce heart failure are of the same order of magnitude as the minimal lethal concentrations for the whole animal variously reported in the literature. Propazone appears to be an exception. According to Stoughton and Baxter (14) 250 mgm. per kgm. of this drug given intravenously to dogs produces very deep anesthesia, and doses between 250 and 350 mgm. per kgm. may produce death. These doses have no significant negative inotropic action on the heart of the dog in the heart-lung preparation and are only small fractions of the dose required to produce a fairly severe heart failure. The relatively low toxicity of propazone to the heart is also apparent from a comparison of its therapeutic and "heart failure" concentrations with those of the other hypnotics in table 1—pentobarbital, chlorobutanol, and paraldehyde. To judge from the data of Stoughton and Baxter propazone is, for example, 1/6 as effective, on a molar basis, as pentobarbital in producing sleep and general anesthesia in dogs. Its negative inotropic action on the dog heart, however, is only 1/30 as strong as that of the barbiturate. On the other hand, the relative potencies of pentobarbital, chlorobutanol, and paraldehyde with respect to their negative inotropic cardiac action and their hypnotic and general anesthetic action are roughly the same.

The increases in pulmonary arterial pressure observed in heart failure induced by the local anesthetics and chlorobutanol can reasonably be attributed only to increases in the resistance of the pulmonary vessels. These changes place an extra burden upon the right ventricle, tax its reserve power, and thus influence unfavorably the outcome of the competence tests. The result is that the drugs which increase resistance in the pulmonary circuit appear to cause a greater depression of the myocardium than is actually the case. The absence of a rise in pulmonary arterial pressure after the administration of paraldehyde is surprising in view of contrary findings reported by Burstein (5) and by Springer *et al.* (6). This point is under further investigation.

#### SUMMARY

Heart failure was produced in the heart-lung preparation of the dog by the administration of the following compounds: the central nervous system depres-

the rate of metabolism had reached a steady level. In this way each vessel served as its own control. In addition, one or two overall controls, containing no drug, were run in each experiment. The rates of metabolism following addition of the drugs were calculated from readings taken during periods of 30 to 60 minutes after a steady level of inhibition had been reached. This usually required 15 to 60 minutes, depending on the drug.

**RESULTS.** The bulk of the experiments was carried out with hearts of guinea pigs. Slices of guinea pig heart muscle respire at a lower rate than slices of dog heart muscle. However, the response to treatment with the drugs studied was found to be, on the whole, quite similar.

*Inhibition of Respiration.* The influence of the various narcotics and local anesthetics on the oxygen uptake of guinea pig heart slices incubated in glucose-containing medium is shown in table 1. The order of potency of these substances differs markedly from that of their negative inotropic action on the heart (cf. 3) in that the narcotics approach or exceed the local anesthetics in their power to inhibit the oxygen uptake of the cardiac slices. Pentobarbital is the most potent inhibitor among the drugs, being effective at a concentration of 0.5 milli-

TABLE 1

*Per cent inhibition of the oxygen uptake of guinea pig heart slices by some narcotics and local anesthetics*

INHIBITOR	CONCENTRATION OF INHIBITOR IN MILLIMOLE PER LITER													
	0.5	1	1.5	2	3	4	6	8	10	20	30	40	80	150
Pentobarbital.....	23	45	60	73	85	92	—	—	—	—	—	—	—	—
Chlorobutanol.....	—	25	38	47	63	76	93	—	—	—	—	—	—	—
Tetracaine.....	—	—	24	59	80	97	—	—	—	—	—	—	—	—
Propazone.....	—	—	—	11	38	54	71	81	87	—	—	—	—	—
Cocaine.....	—	—	—	—	—	—	—	—	2	64	95	—	—	—
Procaine.....	—	—	—	—	—	—	—	—	—	18	49	72	92	—
Paraldehyde.....	—	—	—	—	—	—	—	—	—	14	25	33	56	85

molar. Chlorobutanol and tetracaine follow next in order of potency. Propazone, which was found to have only a weak negative inotropic cardiac action, is a much stronger inhibitor of cardiac respiration than cocaine, which is a highly toxic compound to the heart *in vivo*. Procaine and paraldehyde have to be added in molar concentrations 30 to 60 times that of pentobarbital to produce the same amount of inhibition.

The respiratory quotient (R. Q.) of the cardiac slices, which normally averaged 0.93 during the second hour of incubation, was not changed significantly by the narcotics and local anesthetics.

When the per cent inhibition of respiration by the narcotics and local anesthetics is plotted graphically as a function of their concentration, the resulting curves show that in the low concentration range inhibition increases sharply and nearly linearly with small additions of drug, approaching a limiting value at higher concentrations. Complete inhibition was sometimes obtained at high enough concentrations of drug.

# ACTION OF NARCOTICS AND LOCAL ANESTHETICS ON THE RESPIRATION OF HEART MUSCLE<sup>1</sup>

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Among the various changes which the narcotics produce in living cells, the inhibition of respiratory metabolism has received much attention and has at times been held to be the cause of narcosis<sup>2</sup> (see 1). Particular significance has been attached to the inhibition by narcotics of respiratory processes in the brain (2), partly for the reason that the brain is an organ which cannot tolerate interference with its supply of oxygen without a rapid decline in functional activity. By similar reasoning the depressant action of the narcotics on the heart, another organ which is highly sensitive to anoxia, at least in the warm-blooded animal, might perhaps also be attributed to an inhibition of cellular respiration. In order to throw light on this question, a study has been made of the action of a number of narcotics, and at the same time of several local anesthetics, on the respiration of mammalian heart muscle *in vitro*—i.e., of an action uncomplicated by effects secondary to changes in mechanical activity—and the results have been compared with data (3) on the effect of these agents upon the functional state of the heart, particularly its contractility.

**METHODS.** The experiments were performed with slices of left ventricular muscle of guinea pigs and of dogs. The guinea pigs were young adult males and non-pregnant females weighing 650–800 grams. They were lightly anesthetized with ether, given a blow on the neck, and the heart was quickly excised. The dogs were strong healthy animals in the post-absorptive state. They were anesthetized with ether and bled from the carotid artery previous to the excision of the heart. The ether present in the myocardium of the guinea pigs and dogs had ample chance to escape during the subsequent treatment of the tissue.

The ventricular muscle slices, about 0.6 mm. thick, were prepared and their oxygen consumption was measured according to procedures described or referred to previously (4). Respiratory carbon dioxide evolution was measured by the "direct" method of Warburg. In a series of experiments anaerobic glycolysis was measured. This was done manometrically by the direct method of Warburg in Krebs-Henseleit bicarbonate solution containing 0.2 per cent glucose and in an atmosphere of 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub> freed of oxygen. The rates of oxygen consumption, of respiratory carbon dioxide evolution, and of carbon dioxide evolution due to anaerobic glycolysis are given as cmm. O<sub>2</sub> consumed and cmm. CO<sub>2</sub> evolved per mgm. final dry weight of tissue per hour (Q<sub>O</sub>, Q<sub>CO</sub>, and Q<sub>G</sub><sup>N<sub>2</sub></sup>). The temperature was 38°C. in all experiments. All runs in a given experiment were made with slices obtained from the same heart and were repeated at least twice with slices from different hearts.

The compounds studied were: the narcotics, pentobarbital sodium, chlorobutanol, paraldehyde, and propazone (5,5-di-n-propyl-oxazolidinedione-2,4) sodium; and the local anesthetics, cocaine hydrochloride, procaine hydrochloride, and tetracaine hydrochloride. They were added to the main compartment of the Warburg vessels from the sidearm after

<sup>1</sup>This work was supported by a grant from the Life Insurance Medical Research Fund.

<sup>2</sup>The terms "narcotic" and "narcosis" are used here in the definition of Winterstein (1, p. 13), disregarding their legal and popular connotations.

was complete. Gibson and Long (7) have shown that in dialyzed heart minces pyruvate completely suppresses the endogenous oxygen uptake and that it is oxidized all the way to carbon dioxide and water. *l*-Malate is shown in table 2 to increase the respiration of cardiac slices by about 30 per cent, which is in accordance with the findings of Webb *et al.* (8). The R. Q., however, is not increased when *l*-malate is added, although the theoretical value for its complete oxidation is 1.33. This could mean that oxidation of the malate to oxaloacetate (R. Q. = 0) proceeded at a faster rate than oxidation beyond the oxaloacetate stage. Succinate, in a concentration of 0.1 per cent, causes an immediate sharp rise in oxygen consumption and a simultaneous sharp fall in carbon dioxide production. The R. Q. during the first half hour was 0.10. These data indicate, in agreement with findings of Furchtgott and Shorr (9), that succinate inhibits the normal respiration of heart muscle. From the fact that the increase in oxygen consumption is far greater than the increase caused by *l*-malate and that the latter compound (which in tissues is in equilibrium with fumarate) does not lower carbon dioxide production, it can be inferred that oxidation of succinate to fumarate (R. Q. = 0) was the main oxygen-consuming reaction during the first half-hour following the addition of succinate. The increase in the rate of oxygen consumption caused by 0.1 per cent succinate was found to be followed by a gradual decline to a level somewhat higher than the initial one. This higher level can be obtained more rapidly with smaller concentrations of succinate, after a sharp temporary rise in oxygen consumption which can account for the oxidation to fumarate of all the succinate present. Table 2 gives the respiratory data for the fifth half hour following exposure to  $0.0093 \text{ per cent}$  ( $8 \times 10^{-4} M$ ) succinate in glucose-containing solution. The rate of oxygen uptake was elevated about 15 per cent, the R. Q. was raised slightly to 1.03. This value of the R. Q. might suggest that the increase in respiration was of catalytic nature. However, the total extra oxygen uptake up to the time when the  $Q_{O_2}$  had returned to the control level did not exceed the amount required for the complete oxidation of all the succinate added. It is more likely that the increase in respiration was due to the further oxidation of the succinate originally present beyond fumarate-malate and oxaloacetate. This reaction, when superimposed upon the respiration in glucose, would be expected to produce a slight elevation of the R. Q.

The results in table 2 show that neither glucose or acetate nor pyruvate or *l*-malate are capable of reversing the inhibitory action of pentobarbital on the respiration of the cardiac slices. The R. Q.'s of the slices incubated with these substrates as well as the endogenous R. Q. are not significantly altered by pentobarbital. This suggests that the narcotic does not produce a qualitative change in respiratory metabolism.

Succinate, even in the presence of twice the concentration of pentobarbital used in the other experiments, is able to bring about a very sharp increase in oxygen consumption. Table 2 shows that the  $Q_{O_2}$  rose from 0.6 to 18.0 during the first 30 minutes after the addition of succinate to give a concentration of 0.1 per cent. The control  $Q_{O_2}$  (without pentobarbital) rose from 4.5 to 19.7. The

Comparison of the results in table 1 with data in the literature reveals that the respiration of heart muscle is not much less sensitive to narcotics than the respiration of brain. For example Jowett (5) found that 0.001 M chlorobutanol inhibits the oxygen uptake of guinea pig brain cortex slices incubated in glucose-containing solution by 32 per cent. At this concentration the oxygen uptake of guinea pig heart muscle slices is diminished by 25 per cent.

*Effect of Pentobarbital on Respiration in the Presence of Various Substrates.*—The response of guinea pig heart muscle slices to pentobarbital was studied further in the presence of some non-carbohydrate substrates known to be readily oxidized by this tissue. These substrates were pyruvic acid, acetic acid, succinic acid, and *l*-malic acid. The sodium salts were used. The percentage

TABLE 2

*Effect of pentobarbital on the oxygen uptake and carbon dioxide output of guinea pig heart slices supplied with various substrates*

SUBSTRATE	CONTROL				PENTOBARBITAL, $1.5 \times 10^{-3}$ M				% INHIBITION BY PENTOBARBITAL	
	Before addition of substrate	5-35 min. after addition of substrate			Before addition of substrate	5-35 min. after addition of substrate			Of $O_2$ uptake in presence of substrate	Of increase in $O_2$ uptake by substrate
—	$Q_O_2$	$Q_O_2$	$QCO_2$	R. Q.	$Q_O_2$	$Q_O_2$	$QCO_2$	R. Q.		
Glucose, 0.2%	-4.8	-4.3	3.7	0.86	-1.7	-1.6	1.4	0.87	63	—
Acetate, 0.1%	-5.0	-4.9	4.5	0.92	-1.9	-1.8	1.7	0.95	63	—
Pyruvate, 0.1%	-4.6	-4.1	—	—	-1.6	-1.6	—	—	61	—
Succinate, 0.1%	-4.8	-8.1 <sup>1</sup>	10.0 <sup>1</sup>	1.23 <sup>1</sup>	-1.7	-2.0 <sup>1</sup>	2.4 <sup>1</sup>	1.20 <sup>1</sup>	75	91
<i>l</i> -Malate, 0.114%	-4.5	-19.7	1.9	0.10	-0.6 <sup>2</sup>	-18.0 <sup>2</sup>	0.0 <sup>2</sup>	0.00 <sup>2</sup>	9	0
Succinate, 0.0093% <sup>3</sup>	5.1	-6.6	5.5	0.83	-0.5 <sup>2</sup>	-0.8 <sup>2</sup>	—	0.76 <sup>2</sup>	88	80
	-5.3	-6.1 <sup>4</sup>	6.3 <sup>4</sup>	1.03 <sup>4</sup>	-2.3	-2.6 <sup>4</sup>	2.8 <sup>4</sup>	1.08 <sup>4</sup>	57	62

<sup>1</sup> 50-80 min. after addition.

<sup>2</sup>  $3 \times 10^{-3}$  M pentobarbital.

<sup>3</sup>  $Q_O_2 = -2.5$ ,  $QCO_2 = 1.9$ .

<sup>4</sup> Reaction medium contained 0.2% glucose.

<sup>5</sup> 125-155 min. after addition.

concentrations given are those of the anions. The substrates were added to the main compartment of the reaction vessels from the sidearm after the inhibition of respiration by pentobarbital had become stabilized. The results of these experiments are presented in table 2. Data obtained in the absence of exogenous substrate and with glucose are included for comparison.

Table 2 shows, first of all, in confirmation of the findings of Bernheim and Bernheim (6), that acetate and glucose do not raise the rate of respiration of cardiac slices, while pyruvate has a pronounced effect. The R. Q. in the presence of pyruvate was 1.23. The theoretical value for the complete oxidation of pyruvic acid to carbon dioxide and water is 1.20. The observed value may be taken to indicate that pyruvate was the sole substrate oxidized and that oxidation

Bernheim and Bernheim (6) reported that they found that chloral hydrate and tribromoethanol do not inhibit the oxidation of pyruvate and lactate in rat heart slices. Quastel and Wheatley (10) and Jowett (5) found that chloral hydrate, chlorobutanol, barbiturates and other narcotics do inhibit the oxidation of pyruvate and lactate in rat and guinea pig brain tissue. These authors (11) showed also that the oxidation of these two metabolites in such tissues as liver, kidney, and skeletal muscle is inhibited by narcotics to about the same extent as in brain. From the findings of Bernheim and Bernheim it would appear that there exists a qualitative difference between the pyruvic oxidase systems of heart muscle and of such tissues as brain and skeletal muscle with respect to their behavior toward narcotics. The present results do not suggest such a difference.

*Effect on Anaerobic Glycolysis.* The effect of the narcotics and local anesthetics on the anaerobic glycolysis of guinea pig heart muscle slices was studied at concentrations of these drugs at which oxygen consumption in the presence of

TABLE 3

*Effect of various narcotics and local anesthetics on the anaerobic glycolysis of guinea pig heart slices*

DRUG	CONCENTRATION IN MILLIMOLE/ LITER	$\frac{Q_N}{Q_G}$ BEFORE DRUG	$\frac{Q_N}{Q_G}$ AFTER DRUG	% INHIBITION OF $\frac{Q_N}{Q_G}$ BY DRUG
Pentobarbital.....	3	1.6	1.6	0
Chlorobutanol.....	5	1.6	1.6	0
Propazone.....	8	1.4	1.5	0
Paraldehyde.....	140	1.5	0.9	40
Cocaine.....	25	1.5	1.8	0
Procaine.....	50	1.5	0.7	53
Tetracaine.....	3	1.6	0.4	75

glucose is inhibited 80 per cent. The results are presented in table 3. It is seen that at these concentrations the narcotics, with the exception of paraldehyde, have no significant effect. Such concentrations of narcotics are also without effect on anaerobic glycolysis in brain (12). Tetracaine depresses anaerobic glycolysis to about the same extent as respiration, procaine to a somewhat lesser extent. Cocaine produces a slight stimulation of anaerobic glycolysis. None of the narcotics and local anesthetics was found to inhibit the anaerobic glycolysis of guinea pig and dog heart slices in concentrations producing a moderately severe failure of the isolated dog heart.

Table 3 indicates that the rate of anaerobic acid production in guinea pig heart slices is low. It could not be increased by increasing the phosphate concentration of the suspension medium.

*Comparison of Respiratory and Inotropic Action.* In table 4 a comparison is made between the effect of the various narcotics and local anesthetics on the respiration of dog heart slices incubated in glucose medium and their effect on the contractile power of the dog heart as determined in the preceding commun-

difference in the quotients following succinate may be attributed to the circumstance that in the absence of the inhibitor oxidation of endogenous substrate still continued or that succinate was oxidized further than in the presence of the inhibitor. This is evident from a comparison of the rates of carbon dioxide production. In the control experiment the  $Q_{CO_2}$  fell to 1.9 during the first 30 minutes; in the presence of pentobarbital it became nil. From the  $Q_O_2$  and  $Q_{CO_2}$  values and from the fact that equimolar amounts of *l*-malate do not reverse the inhibition of oxygen consumption by pentobarbital and do not lower carbon dioxide production, it can be inferred that oxidation of succinate to fumarate was the sole oxygen-consuming reaction in the presence of the narcotic and that the rate of the reaction was as rapid as in its absence. When the measurements were continued until the end of the third hour, by which time the  $Q_O_2$  had returned to its initial value of 0.6, almost all the oxygen taken up could be accounted for by the oxidation of all the succinate to fumarate. It is evident from the data presented that pentobarbital has no effect whatever on this reaction. Similar results were obtained with chlorobutanol. Bernheim and Bernheim (6) had found previously that the narcotics chloral hydrate and tribromoethanol are likewise without effect on the oxidation of succinate in heart muscle slices. Thus in heart muscle, like in brain (10, 11), the inhibitory action of narcotics on the respiration is not due to a decrease in the activity of succinic dehydrogenase or that part of the cytochrome system involved in the oxidation of succinate.

The increase in the respiration of the cardiac slices during the fifth half-hour following addition of 0.0093 per cent succinate, which was attributed to the oxidation of this substrate beyond malate and oxaloacetate, is inhibited by pentobarbital (table 2) and also by chlorobutanol to about the same extent as the respiration in glucose and as the increase in respiration caused by *l*-malate. The R. Q. in the presence of pentobarbital was 1.08, which cannot be considered to be significantly different from the control value of 1.03.

It can be seen from table 2 that  $1.5 \times 10^{-3} M$  pentobarbital has a relatively stronger effect on the oxygen uptake in the presence of pyruvate than in the presence of glucose, acetate, or *l*-malate, or than in the absence of added substrate. This is due to the fact that the increase in respiratory activity normally produced by pyruvate is nearly completely prevented by the barbiturate in a concentration which causes only a moderately severe inhibition of the endogenous respiration and of the respiration in the presence of these substrates. The R. Q. rises to about the same value as in the control experiment, namely to 1.20. This may signify that pyruvate is oxidized in preference to endogenous substrate in the presence as well as in the absence of pentobarbital and that the oxidation of whatever small amount of pyruvate is used goes to completion. However, alternative interpretations of the R. Q. are possible. Chlorobutanol was also found to have a relatively stronger inhibitory action on the respiration when pyruvate is used as substrate. The high sensitivity of the pyruvic oxidase system to narcotics may perhaps be partly responsible for the inhibition by these compounds of the oxidation of substrates whose breakdown proceeds via pyruvate.

is well-known (1) that narcotics may stimulate oxidative processes in low concentrations or as an initial effect.

In figure 1 the course of development of myocardial failure in a dog heart-lung preparation, induced by gradually increasing the concentration of pentobarbital, is compared with the corresponding changes in the oxygen consumption of dog heart slices. It is seen that the fall in the rate of oxygen consumption with increasing concentration of the barbiturate proceeds in a fashion different from the decrease in contractility as reflected in the progressive decline of competence and work performance. The most rapid decline of the  $Q_{O_2}$  takes place

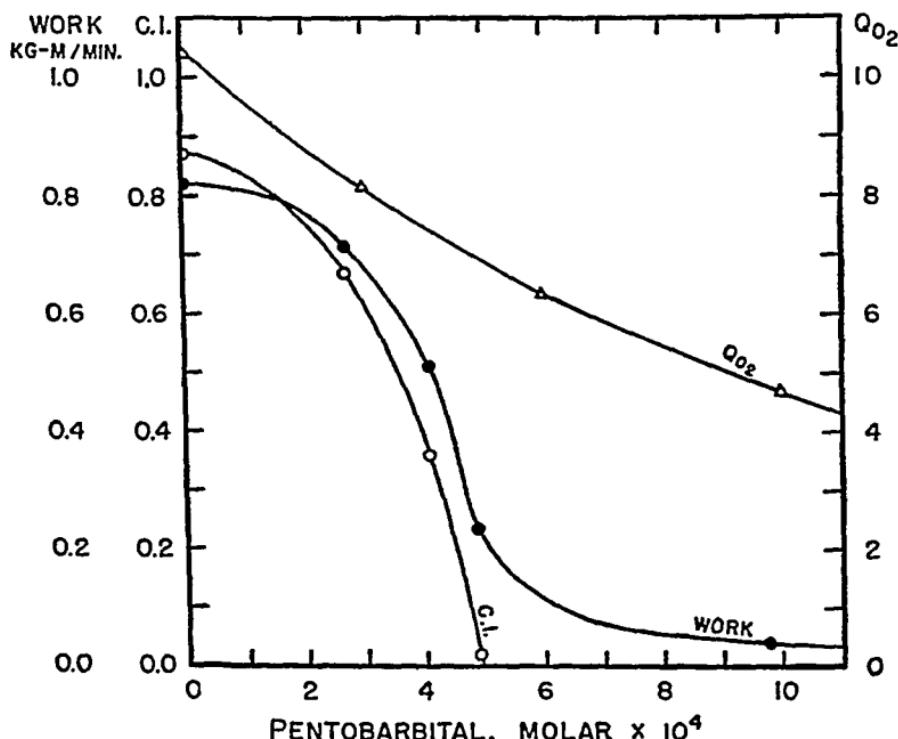


FIG. 1. EFFECT OF PENTOBARBITAL ON THE OXYGEN UPTAKE OF DOG HEART SLICES AND ON THE COMPETENCE INDEX (C.I.) AND WORK PERFORMANCE OF THE DOG HEART

in the low concentration range. Thereafter the curve becomes progressively flatter. In contrast, competence index and work performance are relatively little affected by small amounts of the barbiturate, but decrease very rapidly later on. From these curves one would expect the mechanical efficiency of the heart to be increased by low concentrations of pentobarbital, if the effect of this compound on the respiration of the heart *in vivo* is the same as or similar to its effect on the respiration of cardiac slices. An increase in cardiac efficiency was indeed observed by Gollwitzer-Meier and Krüger (15) in the heart-lung preparation of the dog following administration of small amounts of barbiturates.

cation. The per cent inhibitions of oxygen consumption are listed which are brought about in the heart slices by concentrations of drug producing a fairly severe failure of the isolated dog heart. The table makes it evident that from the standpoint of relative potency *in vitro* and *in vivo* the drugs studied fall into two groups, namely the narcotics on the one hand and the local anesthetics on the other hand. Regardless of their position on the scale of toxicity either *in vitro* or *in vivo*, the narcotics definitely depress the respiration of the cardiac slices in the concentrations used to produce heart failure, while at the corresponding concentrations the local anesthetics have no effect at all.

An analogous difference between representatives of these two groups of compounds has been demonstrated by Larabee, Posternak and Bronk (13) in the nervous system. These authors found that pentobarbital reduces the resting

TABLE 4

*Relative effect of narcotics and local anesthetics on the respiration and on the contractility of heart muscle*

DRUG	CONCENTRATION LOWERING THE COMPETENCE INDEX OF THE DOG HEART FROM 0.9 TO 0.2 millimoles/kgm. dog heart-lung preparation <sup>1</sup>	II % INHIBITION OF THE O <sub>2</sub> UPTAKE OF DOG HEART SLICES BY CONCENTRATION I	
		11.30	55*
Propazone.....	0.45	32	
Pentobarbital.....	17.97	27	
Paraldehyde.....	1.34	23	
Chlorobutanol.....	0.14	0	
Tetracaine.....	1.15	0	
Procaine.....	0.14	0	
Cocaine.....			

<sup>1</sup> Heart, lungs, and blood.

\* Following initial stimulation of 33%.

oxygen consumption of sympathetic ganglia in concentrations which depress, but do not completely block synaptic transmission, while cocaine has no effect on the resting metabolism even in more than 5 times the concentrations in which it blocks transmission. We found that cocaine has no effect on the respiration of cardiac slices even in more than 10 times the concentration in which it produces a moderately severe myocardial failure. It is also of interest that the concentrations of pentobarbital reported by Larabee *et al.* to impair synaptic transmission without producing a complete block are of the same order of magnitude as the concentrations which weaken the contractile power of the heart without abolishing it entirely.

Table 4 shows that the inhibitory action of propazone on the respiration of dog heart slices is considerably stronger than that of the other narcotics and is preceded by a marked increase in activity. Fuhrman and Field (14) have reported that in low concentrations the diphenyl homologue of propazone, but not propazone itself, augments the respiration of rat cerebral cortex slices. It

inotropic action. Indeed, such a contention would be difficult to reconcile with the recent observation (22) that in hearts failing as a result of the action of some of the same narcotic agents used in the present study there is no depletion of adenosine triphosphate and phosphocreatine, the compounds whose energy-rich phosphate bonds are, respectively, the primary source and the immediate reservoir of chemical energy for contraction. Since in these experiments the hearts had been exposed to the narcotics for a period of at least half an hour before a sample was taken for analysis, there can be little doubt that the energy required for the formation of the energy-rich phosphate bonds was furnished primarily by oxidation and not by glycolysis. Hence the impairment of cardiac function could hardly have been the result of an inhibition of oxidative enzymes. The finding that there was an adequate supply of energy-rich phosphates in the presence of concentrations of narcotics which inhibit respiration can be explained on the ground that the energy demand of the narcotized heart is so low that it can be met even though respiration may be markedly reduced.

In his classic monograph "Die Narkose" Winterstein (1) strongly expressed the view that inhibition of respiration is not a specific effect of narcotics and even less a cause of narcosis. He regards it merely as a phenomenon incidental to the general physicochemical changes which the narcotics produce in living systems. Later, Quastel (2, 12) showed that inhibition of respiration by the narcotics is a specific effect and suggested that it is the basis of their general anesthetic action. In the present experiments it was seen that inhibition by narcotics of respiration in the myocardium is a specific effect in the sense indicated by Quastel. However, the inability of these compounds to decrease the energy-rich phosphate store of the heart makes it difficult to regard this inhibition of respiration as a phenomenon of importance so far as the impairment of myocardial contractility is concerned. The conclusion arrived at recently by this author (22) and reached years earlier by Clark *et al.* (21), though on the basis of results differing from those reported here, still stands: The depressant action of the narcotics on the heart is caused by impairment of a phase of myocardial activity other than oxidative metabolism.

#### SUMMARY

1. In concentrations producing a moderately severe failure of the isolated dog heart the narcotics pentobarbital, chlorobutanol, paraldehyde, and propazone markedly reduce the respiration of dog and guinea pig heart muscle slices, while the local anesthetics cocaine, procaine, and tetracaine have no effect even in several times the corresponding concentrations.

2. The increase in the respiration of guinea pig heart slices caused by pyruvate is nearly completely prevented by pentobarbital and chlorobutanol in concentrations which produce only a moderately strong inhibition of respiration in the presence of glucose or acetate or in the absence of added substrate. The R. Q. of guinea pig heart slices incubated in pyruvate is 1.2 and is not changed by pentobarbital. The endogenous R. Q. and the R. Q. in the presence of glucose remain likewise unchanged.

The particular experiment represented in figure 1 was terminated at a final pentobarbital concentration of 1.2 millimolar. At this concentration the  $Q_o$  is lowered to about 4, i.e. the respiration of the cardiac slices is inhibited by about 60 per cent. Nevertheless, the heart was still contracting, though extremely feebly, at a rate of 112 beats per minute.

**DISCUSSION.** For the interpretation of the results it would be of value to know whether the respiration of quiescent cardiac slices is comparable to the resting respiration of the intact heart. According to the estimations of Cohn and Steele (16) the resting oxygen consumption of the dog's heart in the undeteriorated heart-lung preparation is 1.60 cc. per gram heart per hour. In the present experiments the oxygen consumption of dog heart muscle slices during the first 15 minutes of incubation (i.e. at the start of an about 20 per cent decline to a steady level (see 4)) averaged 1.66 cc. per gram initial wet weight of tissue per hour. This value happens to be very close to that given by Cohn and Steele. It may also be noted that the respiratory quotient of the dog heart slices, averaging 0.95 after 2 to 3 hours of incubation in glucose-containing solution, lies just between and close to the values reported by Bayliss, Müller, and Starling (17) and by Cruikshank and Startup (18) for the heart-lung preparation of the dog supplied with glucose.

If the respiration of cardiac slices represents the resting respiration of the intact heart, the results of the present study would be contrary to those of Clark and White (19). These authors found that frog hearts arrested by narcotics continue to respire, even on further addition of drug, at a rate equalling that of the resting unpoisoned organ. They concluded that the resting respiration of the heart, in contrast to the contraction respiration, is resistant to the action of the narcotics, a conclusion in line with a theory of narcosis advanced later by Fisher (20). Clark *et al.* (21) later proposed a different explanation of Clark and White's results by the hypothesis that inhibition of respiration in the myocardium by the narcotics is secondary to inhibition of the contractile process. Gollwitzer-Meier and Krüger (15), on the other hand, working with the heart-lung preparation of the dog, found that doses of barbiturates too small to have a noticeable effect on the circulation may lower the oxygen consumption of the heart. This was taken by them as evidence of a primary inhibition by the barbiturates of oxidative processes in the myocardium, a conclusion corroborated and extended by the present findings. The discrepancies between the findings and conclusions of Clark and collaborators on the one hand and those of Gollwitzer-Meier and Krüger and of the present author on the other hand are probably due to the fact that the former group worked with the frog heart at about 15°C., while the latter employed the mammalian heart at about 38°C. It may be pointed out in this connection that a decrease in temperature lessens the inhibitory action of narcotics on tissue respiration (5, 14), but may fail to diminish the intensity of narcotic action (1).

From the finding that narcotics are able to reduce the respiratory activity of the myocardium independently of a reduction in mechanical activity it does not necessarily follow that the depression of respiration is the cause of their negative

# AN INVESTIGATION OF THE NATURE AND EXTENT OF THE BINDING OF OXOPHENARSINE (MAPHARSEN) BY THE RED BLOOD CELL OF THE RABBIT IN VITRO: I. ADSORPTION ON THE CELL SURFACE<sup>1</sup>

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Wright and Peters (1), in 1943, observed that after the intravenous administration of oxophenarsine to rats, the trypanocidal titer of the blood was at its maximal level immediately after injection, remained so for approximately one hour and then gradually declined so that at eight hours, less than 10 per cent of the blood arsenic was trypanocidally active and after twenty-four hours the amount was only 0.5 per cent. Since the trypanocidal titer of rat's blood decreased markedly between the eighth and twenty-fourth hour in spite of an increase in total blood arsenic, and since Wright (2) had found that this secondary rise in blood arsenic could be attributed to a return of arsenic from the tissues, Wright and Peters concluded that the extra-vascular tissues probably played a major role in the inactivation of oxophenarsine in the body of the rat. Peters (3), in 1942, observed that oxophenarsine underwent a similar but smaller decrease in trypanocidal activity when incubated with rat's blood *in vitro* over periods up to eighteen hours, but was inactivated to only a slight degree when incubated with plasma for the same period of time. These findings led Peters to observe that as much as 80 per cent of the oxophenarsine added to rat's blood was in the erythrocyte fraction, as determined by chemical analysis.

Hogan and Eagle (4), in 1944, noted the relatively close correlation between the therapeutic index of some of the phenyl arsenoxides and the extent to which these compounds bound themselves to the red blood cells *in vitro*. So convinced were these investigators of the great similarity between the binding by blood cells and by the tissues in general of those arsenical compounds which do not change to more toxic compounds in the body that they state "the binding of the phenyl arsenoxides by red blood cells *in vitro* has proved to be a model for their combination with tissues *in vivo* and has so closely paralleled their systemic toxicity as to provide a reliable measure of that factor."

Numerous reports have been published pertaining to the arsenic content of the blood after the administration of various arsenic compounds to experimental animals or man, but the majority of these investigators either did not determine

<sup>1</sup> A summary of the findings herein reported was published in the Federation Proceedings, 4: 143, 1945.

<sup>2</sup> Presented as part of a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota.

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3. From the respiratory data it appears that oxidation of succinate to fumarate in guinea pig heart slices is not inhibited by pentobarbital and chlorobutanol and proceeds at a rapid rate. However, the increase in respiration ascribed to the further oxidation of succinate is strongly inhibited. In the presence of sufficiently large concentrations of succinate and narcotic, carbon dioxide production ceases and almost the whole oxygen uptake can be accounted for by the oxidation of all the succinate to fumarate.

4. None of the above named narcotics and local anesthetics inhibits the anaerobic glycolysis of dog and guinea pig heart slices in concentrations producing a moderately severe failure of the isolated dog heart.

5. The inhibitory action of the narcotics on the respiration of the myocardium is not considered to be the cause of their depressant action on the functional activity of the heart.

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consisted of 0.7 per cent NaCl, 0.2 per cent dextrose, 0.024 per cent KCl and 0.02 per cent anhydrous CaCl<sub>2</sub>, dissolved in freshly glass-distilled water.

The blood was obtained from New Zealand rabbits, of either sex, which were maintained on a diet of Purina Rabbit Chow up to the time of their use. Blood from this species was used because of its ready availability and because, unlike rat's blood (6), its normal arsenic content is very low, an essential feature when experimenting with relatively small amounts of oxophenarsine. The blood was removed directly from the heart of unanesthetised rabbits and 3 mgm. of a crude heparin preparation containing 7.5 cat units per mgm. (Hynson, Westcott and Dunning, Inc.) was added to each cc. of blood.

The procedures used for the preparation of the arsenic-containing samples and the determination of their arsenic content were based upon the official A.O.A.C. Gutzeit method of analysis for arsenic (8). In order to obtain approximately the same acid concentration (15 per cent) in all the samples in the hydrogen generator, the quantity of H<sub>2</sub>SO<sub>4</sub> solution (1 cc. containing 1 gram of H<sub>2</sub>SO<sub>4</sub>) added varied according to the size of the aliquot of the arsenic solution used for the determination. All reagents were as arsenic-free as possible, especially the nitric acid, much of which was redistilled before use.

The amounts of arsenic in the various samples were determined by reference to a graph of standard arsenic stains. This graph was constructed from the mean values of at least ten determinations of the length of stain produced by aliquots containing 0.5 to 10.0 microgm. of arsenic trioxide prepared from a standard arsenic solution containing 1 microgm. of arsenic trioxide per cubic centimeter. A new graph was constructed whenever a different lot of zinc stick was used or a new mercuric bromide solution was prepared. The oxophenarsine content of the sample was calculated from the mean values of 2 to 4 arsenic determinations.

In the adsorption experiments, one part of the appropriately diluted oxophenarsine solution was added to 14 parts of heparinized blood contained in specially-made flat bottom glass tubes. These tubes were then sealed tightly and their contents were mixed thoroughly. They were then fastened to the rotating frame of a specially constructed apparatus that permitted the tubes to make one end-for-end revolution every eight minutes. This rate was sufficient to prevent settling of the cells and did not cause hemolysis. The frame was then immersed in a water bath, the temperature of which was 37.5 ± 0.5°C., and tubes were removed at the end of one-half, four and eight hours. After removal from this apparatus the tubes were centrifuged at 3000 r.p.m. for 30 minutes, the degree of hemolysis was estimated, the cell volume was determined, and a sufficient quantity of plasma was removed from each tube for the arsenic analysis.

**EXPERIMENTAL.** *The in vitro distribution of oxophenarsine between plasma and cells of normal heparinized rabbit's blood at various time intervals and oxophenarsine concentrations.* The final oxophenarsine concentrations in the blood samples were one part in 1, 2, 5, 10, 20, 50, 100, 200 and 500 thousand, respectively. The concentrations beyond 1:50,000 were considered to be within the therapeutic range because it was estimated that the oxophenarsine concentration in the blood of a 70 kgm. patient immediately after the intravenous administration of 60 mgm. of the compound would be approximately 1:83,000. The oxophenarsine solutions were added to the correct volumes of heparinized rabbit's blood and one-half, four or eight hours later, the plasma from each sample was removed and analyzed for its arsenic content. It is assumed that the arsenic remaining in the plasma after the correction for its normal arsenic content is the arsenic of the oxophenarsine molecule and is referred to as such in this paper. Table 1 shows the mean values of the results obtained from at least five experiments conducted at each oxophenarsine concentration and time interval (one exception).

the arsenic distribution between the cells and plasma or serum (5, 6), or the methods of arsenic determination used were not sufficiently sensitive to detect its presence accurately.

Peters (3) determined the distribution of oxophenarsine between the cells and plasma of rat's blood for several different concentrations and found that at the blood oxophenarsine concentration of 1:50,000 sixty per cent of the arsenic was with the red cells immediately after the addition of the compound to the blood; after four hours the figure had increased to 69 per cent and after eight to twelve hours to 81 per cent.

Hogan and Eagle (4) concluded, after determining the extent to which the arsenic was bound by the red cells of oxalated rabbit's blood *in vitro* after three hours incubation, that as the oxophenarsine concentration of the blood was increased, the absolute amount of arsenic bound also increased continuously up to the highest concentrations used, which produced moderate hemolysis of the red cells. The percentage of the total arsenic bound by the red blood cells, however, decreased progressively with increase in the oxophenarsine concentration of the blood, as indicated by the fact that at the lowest oxophenarsine concentration used (1:77,440), ninety per cent of the blood arsenic was bound by the red blood cells while at the highest oxophenarsine concentration (1:300), only 58 per cent of the arsenic was bound by the cells.

Since it appeared from the work of Peters (3) that the binding of an organic arsenic compound by the red blood cells interfered with the ability of the compound to exert its full trypanocidal effect in the blood stream and since relatively little research, other than that of Hogan and Eagle (4), had been conducted to determine the extent of this binding, an investigation of the nature and extent of the binding of oxophenarsine by the red blood cells appeared to be of pharmacological importance. The determination of the *in vitro* binding of oxophenarsine by the red cells was chosen rather than the *in vivo* binding since Wright (2) had observed that there was a continuous exchange of arsenic between the blood and the extra-vascular tissues *in vivo*.

In the studies presented in this paper the extent of the adsorption of oxophenarsine on the red blood cells of the rabbit was determined, followed by an investigation of its degree of reversibility.

**METHODS AND MATERIALS:** A mixture of two lots of oxophenarsine hydrochloride<sup>4</sup> was used in all experiments. Peters (3), using Rodman and Wright's (7) modification of the Treadwell-Hall gravimetric method for the determination of arsenic, found this mixture to contain 28.61 per cent arsenic, a purity of 98.67 per cent. No correction was made for this difference in the arsenic content because in each experiment the calculation of the results from each arsenic analysis was based upon the recovery of oxophenarsine added to a control tube. The sodium salt of oxophenarsine used in all the experiments was prepared immediately before use by adding the required amount of sodium carbonate solution to the hydrochloride before its dilution with the modified dextrose Ringer's solution.

The modified dextrose Ringer's solution was freshly prepared for each experiment and

<sup>4</sup> The oxophenarsine (Mapharsen) was supplied through the courtesy of Parke, Davis and Company.

oxophenarsine concentration of 1:20,000, 53 to 64 per cent at 1:50,000, 37 to 43 per cent at 1:100,000 and 28 to 44 per cent at 1:500,000.

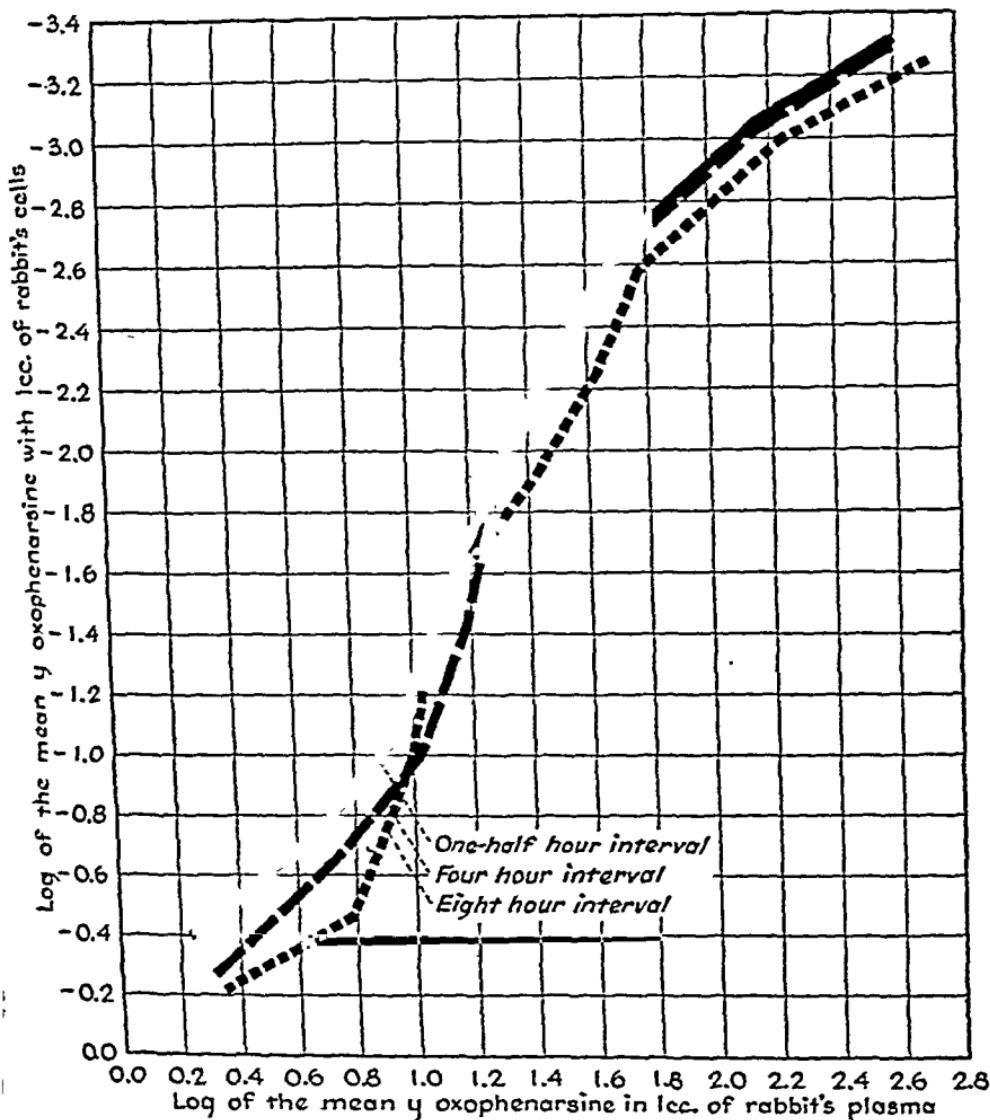


FIG. 1. THE ADSORPTION OF OXOPHENARSINE ON THE RED BLOOD CELLS OF HEPARINIZED RABBIT'S BLOOD AT VARIOUS OXOPHENARSINE CONCENTRATIONS AND TIME INTERVALS

The nature of the attachment of the oxophenarsine to the red blood cell was tested by the application of the Freundlich adsorption isotherm equation (9) to the experimental results; plotting the logarithm of the amount of substance adsorbed by a given weight of adsorbent against the logarithm of the concentra-

It was found that the maximal percentages of oxophenarsine with the cells were attained at the concentrations of 1:2000 and 1:5000; being 80 to 83 per cent and 83 to 85 per cent, respectively. At the concentration of 1:1000 the per cent compound with the cells had decreased to 72 to 76 and although this was probably indicative of beginning saturation of the cell surface, this lower percentage may perhaps be attributed, in part, to the slight hemolysis of the cells, particularly at the four- and eight-hour intervals. The extent of this

TABLE 1

*Summary of the mean values of the distribution in vitro of oxophenarsine at 37.5°C. between the plasma and cells of normal heparinized rabbit's blood at various time intervals and oxophenarsine concentrations*

CONCENTRATION OF OXOPHENARSINE IN THE BLOOD	NO. OF EXPERIMENTS	DURATION OF EXPER. IN HOURS	TOTAL BLOOD VOLUME IN CC.	OXOPHENARSINE IF THE		OXOPHENARSINE PER CC. OF		% OXOPHENARSINE WITH THE CELLS
				Plasma	Cells	Plasma	Cells	
1:1000	5	½	3	752.4	2247.6	396.0	2049.8	74.9
	5	4	3	725.7	2274.3	399.8	1921.5	75.8
	5	8	3	853.1	2146.9	492.1	1695.8	71.6
	5	½	3	275.8	1224.2	145.4	1117.4	81.6
1:2000	5	4	3	255.9	1244.1	141.0	1051.4	82.9
	5	8	3	296.6	1203.4	171.1	951.8	80.2
	5	½	3	100.6	499.4	52.9	455.5	83.2
	5	4	3	92.4	507.6	50.9	429.0	84.6
1:5000	5	8	3	103.6	496.4	59.9	393.0	82.7
	10	½	3	61.9	238.1	32.3	219.2	79.4
	5	4	3	58.2	241.8	32.1	204.5	80.6
	5	8	3	75.9	224.1	44.1	177.7	74.7
1:10,000	10	½	3	42.0	108.0	21.9	99.5	72.0
	5	4	3	37.1	112.9	20.7	94.6	75.3
	5	8	3	45.6	104.4	26.7	83.2	69.6
	10	½	3	24.5	35.5	12.8	32.7	59.2
1:20,000	5	4	3	28.1	31.9	15.5	26.9	53.1
	5	8	3	21.4	38.6	12.4	30.6	64.4
	10	½	3	17.0	13.0	9.0	11.7	43.2
	10	4	3	18.9	11.1	10.2	9.7	37.0
1:50,000	10	8	3	18.4	11.6	10.0	9.8	38.7
	5	½	12	35.5	24.5	4.7	5.5	40.8
	5	4	12	41.3	18.7	5.3	4.4	31.2
	5	8	12	47.5	12.5	6.2	2.9	20.9
1:200,000	5	½	30	33.3	26.7	1.7	2.5	44.5
	5	4	30	40.2	19.8	2.1	1.9	33.0
	5	8	30	43.0	17.0	2.2	1.6	28.4

hemolysis was not sufficient to produce a noticeable effect in the hematocrit reading. Slight hemolysis also occurred at the oxophenarsine concentrations of 1:2000 and 1:5000 during the eight-hour interval, less at the four-hour interval and none at the half-hour interval. The per cent of oxophenarsine with the cells at the lower concentrations decreased progressively with the decrease in the blood oxophenarsine concentration; it was 70 to 75 per cent at the

from curve 1, fig. 2 and table 2. The percentage of the theoretical amount actually released for each concentration and resuspension was then calculated.

It was thus found that the percentage of the theoretical amount of compound actually released for each of the three suspensions was: at the 1:10,000 oxophenarsine concentration 57 per cent for the first suspension, 67 per cent for the second and 71 per cent for the third suspension. At the 1:20,000 concentration the percentages of the theoretical amounts actually released were 67, 70 and 76 and at the 1:50,000 concentration 73, 84 and 90.

TABLE 2

*Summary of the mean values of the results of the five experiments on the distribution in vitro of oxophenarsine between the plasma and cells of heparinized rabbit's blood after repeated resuspensions of the treated cells at one-half hour intervals in fresh plasma\**

ORIGINAL OXOPHENARSINE CONCENTRATION IN THE BLOOD	NUMBER OF TIMES CELLS WERE RESUSPENDED	CPD. ADDED TO THE BLOOD OR ALREADY WITH THE CELLS BEFORE RESUSPENSION	OXOPHENARSINE, AFTER EACH RESUSPENSION IN THE		OXOPHENARSINE, AFTER EACH RESUSPENSION PER CC. OF		TOTAL % CPD. REMOVED BY THE RESUSPENSION
			Plasma	Cells	Plasma	Cells	
1:10,000	Before resuspension	γ	γ	γ	γ	γ	236.5
		600.0	91.6	508.4	23.8	236.5	
		300.0	60.9	239.1	15.8	111.2	
1:20,000	First resuspension	120.0	44.4	75.6	11.5	35.1	8.4
		508.4	50.4	458.0	13.0	214.5	
		239.1	37.9	201.2	9.8	94.2	
1:50,000	Second resuspension	75.6	28.0	47.5	7.3	22.3	12.6
		458.0	40.9	417.0	10.6	195.2	
		201.2	27.9	173.3	7.2	81.1	
1:10,000	Third resuspension	47.5	20.0	27.6	5.2	12.9	21.9
		173.3	29.0	388.1	7.5	182.3	
		31.3	22.2	151.1	5.7	71.0	
1:20,000	Third resuspension	31.3	12.1	19.2	3.1	9.0	40.0
		12.1	—	—	—	—	
		—	—	—	—	—	
1:50,000	Third resuspension	—	—	—	—	—	20.1
		—	—	—	—	—	
		—	—	—	—	—	

\* The total blood volume being 6.0 cc., the temperature 37.5°C.

From this it was concluded that: (a) since the adsorption reaction was from 60 to 90 per cent reversible, adsorption undoubtedly plays the major preliminary role in the binding of oxophenarsine by the erythrocytes; (b) adsorption plays a decreasingly prominent role with increasing oxophenarsine concentration, that is, with increasing saturation of the cell surface; and (c) that factors other than simple adsorption probably enter into the binding of oxophenarsine by the red cells.

DISCUSSION. In the investigation of the distribution of oxophenarsine *in vitro* between the plasma and cells of heparinized rabbit's blood within the oxophenarsine concentration range of from 1:1000 to 1:500,000 and at the time

tion of unadsorbed substance remaining in the liquid phase at equilibrium. If the process is one of adsorption, such a graphic representation should yield a straight line curve.

It was concluded from the results of this plot (see fig. 1) that the oxophenarsine was adsorbed on the red blood cells and that the process was, within certain limits, almost as complete in one-half hour as it was in four or eight hours.

It appears, therefore, that since through the middle range of concentrations (1:5000 to 1:100,000) the curves at all three time intervals are approximately straight lines, within this range of concentrations the chief factor involved in the attachment of oxophenarsine to the erythrocyte is an adsorption of the oxophenarsine on the surface of the red cell.

The deviation of the curves from a straight line at their upper and lower ends may perhaps be explained in part as being due to hemolysis in the higher concentrations and to the diminishing accuracy of the arsenic determinations with very small quantities of arsenic in the lower concentrations.

*The oxophenarsine distribution between the plasma and cells after repeated resuspension of the cells.* The previous experiments having demonstrated that oxophenarsine becomes bound to the red blood cells by a process of adsorption, it appeared desirable to investigate the extent to which this adsorption process was reversible. If no other factor than that of adsorption was involved in this process, then the reaction should be completely reversible and the resuspension of the oxophenarsine-treated cells in fresh plasma should result in the establishment of a new equilibrium corresponding to a point on the original experimental curve appropriate for the total amount of oxophenarsine in the system. Final oxophenarsine concentrations of 1 part in 10, 20 and 50 thousand were employed, these all being concentrations within the range where the adsorption of oxophenarsine strictly follows the Freundlich isotherm.

The procedure was the same as in the previous experiments and one-half hour after the addition of the oxophenarsine to the blood, the plasma was removed and replaced with an equal volume of fresh material. This procedure was repeated twice more, followed by the determination of the arsenic content of each plasma sample.

The mean values of the results of five such experiments are shown in table 2. The method of calculating these results was similar to that used in previous experiments. The curves in fig. 2 were then constructed by the application of the Freundlich adsorption isotherm equation to these experimental results. Curve 1 (fig. 2) was constructed from the mean values of all available data for these initial concentrations.

From the results of these experiments it was concluded that the adsorption of oxophenarsine on the red blood cells was not completely reversible, since the points on curves 2, 3 and 4 (fig. 2) failed to lie approximately along the line of curve 1, which should have been the case had the reaction been completely reversible.

The amount of oxophenarsine that should have been released theoretically by the treated cells, assuming a completely reversible reaction, was calculated

1:5000, the figures being 80 to 83 per cent and 83 to 85 per cent, respectively. At the oxophenarsine concentration of 1:1000 the per cent compound with the cells was from 72 to 76; this decreased binding may have indicated beginning saturation of the cell surface although there was also slight hemolysis of the cells at this concentration. The amount of compound bound by the red blood cells at oxophenarsine concentrations of less than 1:5000 decreased progressively with decreasing oxophenarsine concentration in the blood sample so that at the concentration of 1:500,000 only 28 to 44 per cent of the compound was with the red blood cells. These results are in agreement with those obtained by Thuret (10) using defibrinated horse blood and oxophenarsine concentrations greater than 1:20,000, but the results obtained by Hogan and Eagle (4) with oxalated rabbit's blood were from one-tenth to one-third higher than those reported here at corresponding blood oxophenarsine concentrations; this difference may possibly be attributed to the use of oxalate instead of heparin as the anticoagulant.

It was also observed that within the oxophenarsine concentration range of from 1:5000 to 1:100,000, the adsorption curves at the three time intervals (1/2, 4 and 8 hours) were approximately straight lines when plotted according to the Freundlich adsorption isotherm equation. Adsorption, no doubt, also played a major role beyond these concentrations despite the fact that the curves deviated from their straight course. Further, it was concluded that the extent of the adsorption was almost as complete in one-half hour as in four or eight hours. This rapid rate of adsorption of oxophenarsine by the red blood cell is in agreement with the results obtained by Thuret, but Hogan and Eagle found the binding to be complete only after two hours; this difference in the results may perhaps be attributed to the fact that these latter workers did not continuously mix the blood samples during the adsorption period.

The adsorption of oxophenarsine on the rabbit's red blood cells was not completely reversible when the oxophenarsine-containing cells were resuspended three times in fresh plasma although the per cent oxophenarsine not liberated by the cells decreased progressively after each resuspension. The degree of reversibility of the reaction was in most cases greater than 60 per cent for the first resuspension and in the case of the lowest oxophenarsine concentration used approached as high as 90 per cent. Thuret observed the adsorption of oxophenarsine on the red blood cell of defibrinated horse blood to be completely reversible.

#### CONCLUSIONS

An investigation of the nature and extent of the binding of oxophenarsine by the red blood cell of rabbit *in vitro* showed that:

1. Through the oxophenarsine concentration range of from 1:1000 to 1:500,000, from 28 to 85 per cent of the oxophenarsine added to heparinized rabbit's blood was bound by the red blood cells in one-half, four or eight hours. The extent of the binding was almost as complete in one-half hour as after four or eight hours. The oxophenarsine bound by the cells increased with increasing oxophenarsine concentration to a maximum of 85 per cent at the concentration of 1:5000.

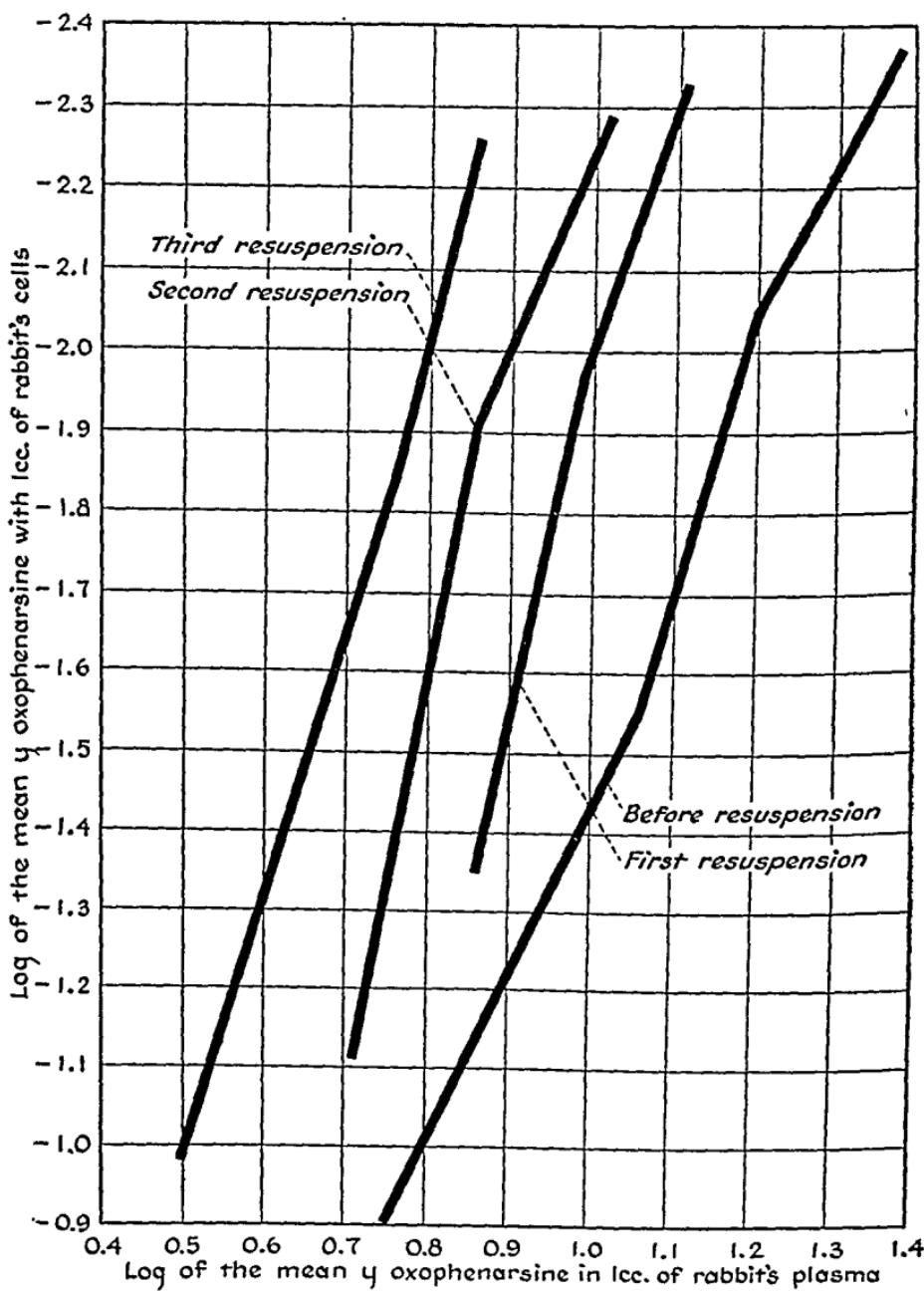


FIG. 2. THE REDISTRIBUTION OF OXOPHENARSINE BETWEEN RABBIT'S RED BLOOD CELLS AND PLASMA AFTER THE TREATED CELLS WERE REPEATEDLY RESUSPENDED AT ONE-HALF HOUR INTERVALS IN FRESH PLASMA

intervals of 1/2, 4 and 8 hours, it was found that the maximal percentages of oxophenarsine with the cells were attained at the concentrations of 1:2000 and

# AN INVESTIGATION OF THE NATURE AND EXTENT OF THE BINDING OF OXOPHENARSINE (MAPHARSEN) BY THE RED BLOOD CELL OF THE RABBIT *IN VITRO*: II. FACTORS OTHER THAN ADSORPTION<sup>1</sup>

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It was shown in a previous publication (1) that from 28 to 85 per cent of the oxophenarsine added to rabbit's blood *in vitro* is bound to the red cells. This binding of the oxophenarsine is chiefly in the nature of an adsorption on the cell surface and follows the requirements of the Freundlich isotherm equation over a wide range of concentrations.

The reaction is not, however, completely reversible as determined by the amount of oxophenarsine removed from the cells when repeatedly resuspended in fresh plasma; this suggests that reactions secondary to the adsorption also play a role in the binding of the oxophenarsine.

A number of factors may have been involved in the incompleteness of the reversibility of this adsorption reaction. First, the oxophenarsine may have combined firmly with some component on the cell surface. This was a possibility because Newton, Benedict and Dakin (2) in 1927, isolated the sulphydryl compound, thiasine, from the red blood cell membrane and Rosenthal (3), in 1932, observed that oxophenarsine would combine with the free sulphydryl groups of proteins. Second, the oxophenarsine may be first adsorbed on the cell surface followed by its diffusion into the cell yet fail to combine with some component inside the cell. If this were true, however, a re-establishment of the equilibrium during each resuspension of the cells in fresh plasma would only be delayed depending upon the rate at which the oxophenarsine passed through the cell membrane. It was assumed, however, that the one-half hour period of resuspension was sufficiently long to permit the re-establishment of equilibrium because of the rapid rate of adsorption of oxophenarsine on the red blood cell. A third factor that may have been involved was that a fraction of the oxophenarsine may have passed into the interior of the red blood cell after its preliminary adsorption and there have combined firmly with some cell component, which would result in the failure to re-establish equilibrium between the plasma-cell phase during the resuspension period. This appeared quite possible since Hunter, Kip and Irvine (4) had discovered that 95 per cent of the radio-active potassium arsenite present in the rat's blood after its administration was with

<sup>1</sup> A summary of the findings herein reported was published in the *Federation Proceedings*' 4: 143, 1945.

<sup>2</sup> Presented as part of a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota.

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2. Adsorption played the major role in the binding of oxophenarsine within the concentration range of 1:5000 to 1:100,000 as evidenced by substantial adherence to the Freundlich adsorption isotherm equation. Even at the highest oxophenarsine concentration used, the surface of the red blood cells showed little evidence of being saturated.

3. The degree of reversibility of this adsorption process, as determined by three resuspensions of the oxophenarsine-treated cells in fresh plasma, was in all cases greater than 60 per cent after a single resuspension and in some cases approached as high as 90 per cent after three resuspensions.

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part of the oxophenarsine solution. All of the samples were fastened to the frame of the rotating apparatus and immersed in the water bath ( $37.5 \pm 0.5^{\circ}\text{C}$ .) for thirty minutes (see previous article). Five cc. of each sample were then transferred to a collodion membrane which, in turn, was suspended in a large tube that contained 45 cc. of modified dextrose Ringer's solution; this quantity of solution was sufficient so that the fluid levels on each side of the membrane were equal. Small glass stirrers mixed the contents on each side of the collodion membrane; the pulleys, which were connected to the stirrers inside the collodion membranes, were of a larger diameter in order to decrease the rate of stirring of the contents, partly because of the smaller volume, as compared to that of the large tube and also to reduce hemolysis to a minimum. The apparatus was placed in an incubator, the temperature of which was approximately  $37^{\circ}\text{C}$ . The solution in the large chamber was replaced with fresh modified dextrose Ringer's solution every three hours for the first twelve hours; the outside of the collodion membrane was washed thoroughly with the Ringer's solution each time. At the end of either twelve or twenty-four hours, the material inside the collodion membrane, the membrane itself and the solution in the chamber were analyzed separately for their arsenic content; the solutions from the third and sixth hour periods were pooled and analyzed for their arsenic content, likewise those from the ninth and twelfth hour periods. A control sample of defibrinated rabbit's blood was also prepared with the modified dextrose Ringer's solution and was not only used for the determination of the normal arsenic content, but an aliquot was placed in a collodion membrane and at the end of the experiment, the solution in the chamber was tested for the presence of protein.

In the diffusion experiments with the laked defibrinated blood the various samples of defibrinated rabbit's blood were prepared in the same manner as described previously, with the exception of the addition of saponin. After laking the cells, the oxophenarsine was added and the remainder of the experiment was similar to the ones mentioned previously; saponin was likewise added to the serum as a control.

In the diffusion experiments with the hemoglobin solution, 6.5 parts of modified dextrose Ringer's solution was added to 7.5 parts of the specially prepared hemoglobin solution followed by 1 part of the oxophenarsine stock solution. In the experiments with the stroma which were prepared from 5.6 cc. of defibrinated rabbit's blood they were suspended in 14 parts of modified dextrose Ringer's solution, 1 part of the oxophenarsine solution was added and 5 cc. of this material was dialyzed.

The procedures used for the preparation of the arsenic-containing samples and the determination of their arsenic content were the same as in the first paper (1). It is assumed, as was previously, that the arsenic remaining in the various samples after the correction for the normal arsenic content is the arsenic of the oxophenarsine molecule, and it is referred to as such in this paper.

**EXPERIMENTAL.** Previous experiments having demonstrated that the adsorption of oxophenarsine on erythrocytes was not completely reversible, it appeared possible that in addition to the intact cell membrane (adsorption), certain cell constituents might play a role in fixing the oxophenarsine.

For this reason the ability of oxophenarsine to dialyze from a blood suspension was determined both in the presence of the intact cell membrane (normal erythrocytes) and after complete removal of the cell membrane (laked blood).

Experiments were first conducted to determine the extent to which oxophenarsine, when dissolved in modified dextrose Ringer's solution, would dialyze through the collodion membrane. Rosenthal (3) had shown that oxophenarsine behaved as a crystalloid and passed quantitatively through a collodion membrane under ultrafiltration. In our experiments, oxophenarsine was dialyzed using a

the red blood cells; 62 per cent of it was found in the globin fraction of hemoglobin and the remainder with the heme fraction.

The role played by these various factors other than adsorption on the cell surface was investigated, therefore, by determining the diffusibility of oxophenarsine when subjected to dialysis from preparations containing normal blood, laked blood, hemoglobin or other blood component.

**METHODS.** The sample of oxophenarsine and the preparation of its solutions was the same as that mentioned in the previous report (1).

The source of the rabbit's blood was the same as that in the previous paper (1). The defibrinated blood was prepared in the usual manner and filtered through several thicknesses of gauze before use. The laked defibrinated blood samples were prepared by adding 7.5 mgm. of saponin to each cc. of defibrinated blood. In the experiments with the defibrinated blood, the cell volume, hemoglobin content and the normal arsenic content of the blood and serum were determined on an aliquot of the blood sample diluted to the same extent as the samples containing the oxophenarsine.

The hemoglobin solution was prepared from a single lot of defibrinated rabbit's blood. This blood was centrifuged and the cells were repeatedly resuspended in an equal volume of 1 per cent NaCl solution previously cooled to approximately 5°C. The washed cells were then transferred to a moistened cellophane tube which was clamped tightly in order to prevent volume changes and placed in running tap water for twenty-four hours. The material was then centrifuged to remove the stroma and placed in the freezing unit of the refrigerator until needed. The hemoglobin content of this solution was found to be 22.50 grams/100 cc. as determined by the Coleman photoelectric colorimeter and its normal arsenic content was determined from two aliquots of the final preparation. This yielded a concentrated hemoglobin solution which, after the addition of the modified dextrose Ringer's solution, had a final concentration approximately equal to that of normal rabbit's blood.

The suspensions of cell stroma were prepared by taking the same amount of defibrinated blood used in the dialysis experiments (5.6 cc.) centrifuging it and repeatedly resuspending the cells in 1 per cent NaCl solution. Five cc. of distilled water were then added and after four resuspensions in the NaCl solution, the stroma were stored in the freezing unit of the refrigerator until needed. The normal arsenic content of these suspensions was determined on one of the duplicate samples in each experiment.

Powdered urease (E. R. Squibb and Sons), originally prepared from the Jack Bean (*Cananalia ensiformis*) was added to modified dextrose Ringer's solution so that its final concentration was between 12 and 13 per cent.

The modified dextrose Ringer's solution was freshly prepared for each experiment and consisted of 0.7 per cent NaCl, 0.2 per cent dextrose, 0.024 per cent KCl and 0.02 per cent anhydrous CaCl<sub>2</sub>, dissolved in freshly glass-distilled water.

The collodion membranes were prepared from a solution of 4 per cent nitrocellulose dissolved in equal parts of absolute alcohol and dry ethyl ether.<sup>4</sup> The thickness of these membranes was from 155 to 185 microns and was maintained as uniform as possible by applying the three layers of collodion solution to the horizontally rotated, specially constructed glass tubes in the same manner each time. After thorough washing, the membranes were stored in distilled water in the refrigerator; new membranes were prepared every four weeks.

In all of the diffusion experiments, the oxophenarsine concentration inside the collodion membrane at the beginning of dialysis was 1:10,000. The samples of defibrinated rabbit's blood or serum containing the oxophenarsine consisted of 14 parts of blood or serum and 1

<sup>4</sup> Grateful appreciation is expressed to Dr. K. Sollner, formerly of the Department of Physiology, for advice in the preparation of the membranes, and for permission to use his special apparatus for this purpose.

was carried out to compare the dialyzability of oxophenarsine (a) in the presence of the intact red cell membrane, that is, in normal defibrinated blood and (b) in the absence of the red cell membrane, that is, in laked defibrinated blood.

Oxophenarsine was added to the samples in a final concentration of 1:10,000; the tubes were placed in the rotating apparatus in the water bath for one-half hour to obtain equilibrium of the adsorption reaction, then placed in the collodion membranes. Preliminary experiments having shown that only from 1 to 5 per cent additional oxophenarsine dialyzed between the 12th and 24th hours the experiments employing cellular media were terminated after 12 hours to avoid hemolysis.

Using defibrinated rabbit's blood, 33 per cent of the added oxophenarsine dialyzed in the first six hours and 48 per cent in twelve hours. Since the direct adsorption experiments described previously (1) had shown that at this concentration of oxophenarsine 80 per cent of the compound was bound to the red cells and 20 per cent was in the serum, and since it has been further shown that serum does not bind oxophenarsine, it may be deduced that the 48 per cent of the added oxophenarsine that passed through the membrane in 12 hours was composed of the 20 per cent dialyzable compound normally free in the serum plus an additional 28 per cent reversibly attached to the red cells. The remaining 47 per cent (95-48) of the oxophenarsine was firmly attached to the red cells in such a manner as to prevent its passage through the collodion membrane during the experimental period.

The influence that the intact red blood cell membrane exerted on the extent of the dialysis of oxophenarsine through the collodion membrane from blood was determined by laking the red blood cells with saponin before the addition of the oxophenarsine. In these experiments with laked defibrinated blood, 59 per cent of the oxophenarsine diffused in six hours and 75 per cent in twelve hours. These percentages were higher than those obtained with the defibrinated normal blood (33 and 48 per cent respectively) at the corresponding time intervals, but were less than those obtained with the saponin-containing serum (92 and 97 per cent respectively).

Since 75 per cent of the added oxophenarsine dialyzed in 12 hours from the laked defibrinated blood and only 48 per cent from the corresponding non-laked sample, it may be assumed that the difference of 27 per cent may be attributed to oxophenarsine irreversibly bound to the cell membrane. Any oxophenarsine which might have penetrated the cell membrane but remained uncombined with any of the cell constituents, yet failed to return to the serum fraction during the dialysis period would also be included in what has been designated as the role played by the intact cell membrane.

Since approximately 20 per cent (95-75) of the added oxophenarsine still failed to dialyze through the collodion membrane from laked defibrinated blood, it appeared probable that additional amounts of oxophenarsine might also be bound by other cell components such as hemoglobin or the cell stroma.

Good theoretical grounds existed for assuming that hemoglobin might play a role in the binding of oxophenarsine, since Mirsky and Anson (5) had shown

collodion membrane and a volume ratio of 1:9 inside and outside the membrane. The solution outside the membrane was replaced at the 6th and 12th hours with fresh modified dextrose Ringer's solution. It was found that from 63 to 69 per cent (mean 66.0) of the oxophenarsine dialyzed in 6 hours, 90 to 93 per cent (mean 91.7) in 12 hours and 97 to 98 per cent (mean 97.6) dialyzed in 24 hours (see table 1).

Experiments were next carried out to determine the extent of dialysis of oxophenarsine from rabbit serum, the solution outside the membrane in the remaining experiments being replaced every three hours for the first twelve hours. It was found that 90 per cent of the oxophenarsine dialyzed in 6 hours and 96 per cent in 12 hours. Since these figures are at least as great as those obtained for the dialysis of oxophenarsine from a dextrose Ringer's solution it may be concluded that the proteins of rabbit plasma or serum do not bind oxophenarsine.

TABLE 1

*Per cent oxophenarsine dialyzing through collodion membranes from various media after a preliminary mixing period of 30 minutes at 37°C.; the modified dextrose Ringer's solution in the dialyzing chamber was replaced every three hours*

MEDIUM INSIDE THE COLLODION MEMBRANE	NUMBER OF EXPERIMENTS	MEAN PER CENT OF TOTAL OXOPHENARSINE DIALYZING IN	
		6 hours	12 hours
Modified dextrose Ringer's solution . . .	2	66.0	91.7
Rabbit serum	5	90.4	95.5
Rabbit serum plus saponin	3	91.8	96.8
Defibrinated rabbit blood	5	33.1	47.6
Laked defibrinated rabbit blood	3	59.1	75.1
*15% rabbit hemoglobin solution . . .	2	71.1	81.7
*13% urease solution	2	41.4	56.6
*Cell stroma of rabbit blood	2	86.4	93.0

\* In Modified Dextrose Ringer's Solution.

Oxophenarsine concentration in media before dialysis was 1:10,000; the volume ratio of media to solution outside membrane was 1:9. Temperature during dialysis was 37°C.

These results also agree with those of Rosenthal (3) who found that oxophenarsine passed quantitatively through a collodion membrane from rabbit serum when subjected to ultrafiltration.

Similar tests were made using serum to which saponin had been added in the same concentration as that used to produce laking of the red cells. It was found that 92 per cent of the oxophenarsine dialyzed in 6 hours and 97 per cent in 12 hours. It was concluded, therefore, that saponin did not interfere with the passage of oxophenarsine across the collodion membrane. The mean rate of dialysis for the above three control media was 83 per cent in 6 hours and 95 per cent in 12 hours.

Having established in these preliminary experiments that oxophenarsine is 95 per cent dialyzable through a collodion membrane from modified dextrose Ringer's solution, serum and serum containing saponin, a series of experiments

5. The amount of oxophenarsine bound by the stroma was within the limits of experimental error.

6. The role of the free sulphhydryl groups of proteins in the binding of oxophenarsine was further substantiated by demonstrating the lack of binding of oxophenarsine by serum, its moderate binding by hemoglobin and its extensive binding by urease.

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that approximately 28 per cent of the total number of sulphydryl groups in horse hemoglobin were in the free form at the pH of the blood and because Rosenthal (3) concluded that approximately 90 per cent of the oxophenarsine added to heat coagulated egg white combined with the free sulphydryl groups of the protein molecule. Hunter, Kip and Irvine (4) had also observed that after the administration of radio-active potassium arsenite to rats, 95 per cent of the material in the blood was bound to the hemoglobin.

A concentrated hemoglobin solution was so prepared that, when diluted with the modified dextrose Ringer's solution, the final hemoglobin concentration was approximately equal to that of defibrinated normal blood. After the addition of the oxophenarsine two experiments were conducted in the same manner as those mentioned previously. It was found that 71 per cent of the oxophenarsine passed through the membrane in six hours and 82 per cent in twelve hours. Since previous experiments demonstrated that 95 per cent of the oxophenarsine dialyzed from serum and 75 per cent from laked defibrinated blood, it was concluded that 13 per cent of the approximately 20 per cent of oxophenarsine that failed to dialyze from laked blood could be accounted for by binding on the hemoglobin.

In order to test further the binding of oxophenarsine by free sulphydryl groups, experiments were carried out to determine the extent to which oxophenarsine was bound by urease, a natural protein known to possess a large number of free sulphydryl groups (6). When oxophenarsine was dialyzed from a 14 per cent urease solution (corresponding to the hemoglobin concentration) it was found that 41 per cent dialyzed in 6 hours and 57 per cent in 12 hours. The binding of oxophenarsine by urease is, therefore, almost three times as great as the binding by an equivalent amount of hemoglobin.

The binding of oxophenarsine by the stroma of rabbit's red blood cells was determined by adding the oxophenarsine to samples of stroma prepared from an equivalent amount of defibrinated blood used in the previous dialysis experiments. With this stroma preparation, 86 per cent of the oxophenarsine dialyzed in six hours and 93 per cent in twelve hours. It was concluded that the extent of binding of oxophenarsine by the stroma was only slight and not sufficiently different from that of serum to be sure that the numerical differences represented a statistically significant difference.

#### CONCLUSIONS

An investigation of the dialysis of oxophenarsine through the collodion membrane from various media prepared from rabbit blood and urease showed that:

1. Approximately 95 per cent of the oxophenarsine dialyzed from serum.
2. Of the 48 per cent oxophenarsine that dialyzed from defibrinated blood, 20 per cent represented the amount present in the serum and 28 per cent was the oxophenarsine reversibly bound to the cell surface.
3. An additional 27 per cent oxophenarsine dialyzed when the intact cell membrane was destroyed with saponin.
4. The hemoglobin accounted for 13 per cent of the oxophenarsine bound by the laked defibrinated blood.

## INDEX

- Acetanilide and its metabolic products, Estimation of, in biological fluids and tissues, 22  
Fate of, in man, 29
- Adrenergic blocking agents, Effect of, on vasoconstriction produced by acute oxygen lack, 221
- nervous reflexes, epinephrine and nicotine, pressor responses to, Effect of two halogenated ethylamines on, 350
- Alles, Gordon A., and Ellis, Charles H. Comparative actions of certain compounds like alpha-fagarine, 416
- Alpha-fagarine and similar compounds, Comparative actions of, 416
- Aminophylline and diphenhydramine, Protective action of combinations of, in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367
- Analgesic action of methadon isomers and other analgesics, Evaluation of, by new rapid screening method, 328
- Anesthesia. XXXI. Study of cyclic and noncyclic hydrocarbons on cardiac automaticity, 315
- Anesthetics, Comparison of nine local, 299 local, and central nervous system depressants, Experimental heart failure caused by, 439  
local, and narcotics, Action of, on respiration of heart muscle, 444
- Angistomin and other substances, Influence of tetraethylammonium on responses of isolated intestine to, 244
- Antifibrillatory action of coronary dilator drugs, 136
- Antihistamine and bronchodilator drugs, Response to, of anaphylactic guinea pig trachea, 412
- Antihistaminic drugs, Potentiating effect of, on sedative action of barbiturates, 7
- Arterial pressure, Role of, in induction of idioventricular rhythms under cyclopropane anesthesia, 319
- Atropine antagonism, Lack of, to convulsant action of strychnine, 78
- Axelrod, Julius, and Brodie, Bernard B. Estimation of acetanilide and its metabolic products in biological fluids and tissues, 22  
and Brodie, Bernard B. Fate of acetanilide in man, 29
- BAL, Inability of, to prevent or modify acute renal injury induced by uranium nitrate, 262
- Barbiturates, determination of, Ultraviolet spectrophotometric procedure for, 68  
sedative action of, Potentiating effect of antihistaminic drugs on, 7
- Bernheim, Frederick, Fitzgerald, Dorothea B., and Eadie, George S. Inhibition of cholinesterase of rat brain by methadon, 19
- Beyer, Karl H., Tillson, Elizabeth K., Russo, Horace F., Schuchardt, Grace S., and Gass, S. Richard. Absorption and elimination of caronamide, 167
- Blood cell, red, of rabbit, Binding of mapharsen in, *in vitro*, 455, 462
- Bodansky, Oscar, and Finkelstein, Murray. Effect of cardiac glycosides on respiration of cardiac muscle, 274
- Bope, Frank W., Cranston, Elizabeth M., and Gisvold, Ole. Tannin obtained from *Pinus Caribaea*, Morelet, 209
- Boyd, Beverly L., Josephson, Edward S., and Greenberg, Joseph. Synergistic effect of chlorguanide and sulfadiazine against *Plasmodium gallinaceum* in chick, 60
- Brodie, Bernard B., and Axelrod, Julius. Estimation of acetanilide and its metabolic products in biological fluids and tissues, 22  
and Axelrod, Julius. Fate of acetanilide in man, 29  
and Flinn, Frederick B. Effect on pain threshold of N-acetyl p-aminophenol, 76
- Lief, Philip A., and Poet, Raymond. Fate of procaine in man following intravenous administration and methods for estimation of procaine and diethylaminoethanol, 359
- Bromal hydrate and chloral hydrate: pharmacological contrast and its chemical basis, 401



- Dearborn, Earl H. Comparison of one dose per day with six doses per day of quinine in suppression of lophuriae malaria in duck, 178
- Diethylaminoethanol and procaine, Methods for estimation of, 359
- Digitoxin and histamine, Study of pharmacological relationship between, 53
- Diphenhydramine and aminophylline, Protective action of combinations of, in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367
- Eddie, George S., Bernheim, Frederick, and Fitzgerald, Dorothea B. Inhibition of cholinesterase of rat brain by methadon, 19
- Ellis, Charles H., and Alles, Gordon A. Comparative actions of certain compounds like alpha-fagarine, 416
- Ellis, Sydney. Enzymic hydrolysis of morphine esters, 130
- Emetine, Toxicity, distribution and excretion of, 431
- Enzymic hydrolysis of morphine esters, 130
- Epinephrine and histamine, antagonism with N-(2-haloalkyl)-1-naphthalene-methylamine derivatives, 339
- and histamine, Influence of lung distension on response of bronchiles to, 182
- nicotine and adrenergic nervous reflexes, pressor responses to, Effect of two halogenated ethylamines on, 350
- Epstein, Herbert J., Krementz, Christie S., and Cahen, Raymond L. Evaluation of analgesic action of methadon isomers and other analgesics by new rapid screening method, 328
- Ethylamines, Effect of two halogenated, on pressor responses to epinephrine, nicotine and adrenergic nervous reflexes, 350
- Ferguson, J. K. W., Hamilton, H. S., and Westfall, B. A. Comparison of nine local anesthetics, 299
- Fibrillation, ventricular, hydrocarbon-epinephrine induced, Studies on, 12
- Fieser, Louis F., and Heymann, Hans. Naphthoquinone antimalarials. XIX. Antirespiratory study of protein binding, 97
- Chang, Frederic C., Dauben, William G., Heidelberger, Charles, Heymann, Hans, and Seligman, Arnold M. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Heymann, Hans, and Seligman, Arnold M. Naphthoquinone antimalarials. XX. Metabolic degradation, 112
- Fink, Lewis D., and Wright, Harold N. Binding of mapharsen by red blood cell of rabbit *in vitro*, 455, 462
- Finkelstein, Murray, and Bodansky, Oscar. Effect of cardiac glycosides on respiration of cardiac muscle, 274
- Fitzgerald, Dorothea B., Eddie, George S., and Bernheim, Frederick. Inhibition of cholinesterase of rat brain by methadon, 19
- Flinn, Frederick B., and Brodie, Bernard B. Effect on pain threshold of N-acetyl p-aminophenol, 76
- p-Fluorophenethylamine and p-fluorophenethyl-methylamine, Comparative pharmacology of, 192
- Frank, Karl, and Wikler, Abraham. Hind-limb reflexes of chronic spinal dogs during cycles of addiction to morphine and methadon, 382
- Freese, Homer B., Winbury, Martin M., Michiels, Patricia M., and Hambourger, W. E. Protective action of combinations of diphenhydramine and aminophylline in fatal bronchospasm in guinea pigs due to mecholy aerosol, 367
- Freyburger, Walter A., Moe, Gordon K., Malton, S. Donald, and Rennick, Barbara R. Role of arterial pressure in induction of idioventricular rhythms under cyclopropane anesthesia, 319
- Garb, Solomon, and Chenoweth, Maynard B. Studies on hydrocarbon-epinephrine induced ventricular fibrillation, 12
- Gass, S. Richard, Beyer, Karl H., Tillson, Elizabeth K., Russo, Horace F., Schuchardt, Grace S. Absorption and elimination of caronamide, 167
- Giarmani, Nicholas J. Antibiotic lactones and synthetic analogs. I. Cardiotoxic effects on isolated frog heart, 232
- Gimble, Abraham I., Davison, Clarke, and Smith, Paul K. Toxicity, distribution and excretion of emetine, 431
- Gisvold, Ole, Bope, Frank W., and Cranston, Elizabeth M. Tannin ob-

- Bronchioles, response of, to epinephrine and histamine, Influence on, of lung distention, 182
- Bronchodilator and antihistamine drugs, Response to, of anaphylactic guinea pig trachea, 412
- Bronchospasm, fatal, in guinea pigs, due to mecholyl aerosol, Protective action of combinations of diphenhydramine and aminophylline in, 367
- Bruce, Margaret D., MacNider, Wm. deB., and Trott, Jesse C., Jr. Inability of BAL to prevent or modify acute renal injury induced by uranium nitrate, 262
- Butler, Thomas C. Bromal hydrate and chloral hydrate: pharmacological contrast and its chemical basis, 401
- Cahen, Raymond L., Epstein, Herbert J., and Krementz, Christie S. Evaluation of analgesic action of methadon isomers and other analgesics by new rapid screening method, 328
- Caplan, H., and Melville, K. I. Influence of lung distention on response of bronchioles to epinephrine and histamine, 182
- Cardiac automaticity, Study of cyclic and noncyclic hydrocarbons on, 315
- muscle, respiration of, Effect on, of cardiac glycosides, 274
- Cardiotoxic effects on isolated frog heart, of antibiotic lactones and synthetic analogs, 232
- Caronamide, Absorption and elimination of, 167
- Carr, C. Jelleff, Vitcha, James F., and Krantz, John C., Jr. Anesthesia. XXXI. Study of cyclic and noncyclic hydrocarbons on cardiac automaticity, 315
- Castillo, Julio C. Anaphylactic guinea pig trachea and its response to antihistamine and bronchodilator drugs, 412
- Central nervous system depressants and local anesthetics, Experimental heart failure caused by, 439
- Chang, Frederic C., Dauben, William G., Heidelberger, Charles, Heymann, Hans, Seligman, Arnold M., and Fieser, Louis F. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products. 85
- Chenoweth, Maynard B., and Garb, Solomon. Studies on hydrocarbon-epinephrine induced ventricular fibrillation, 12
- Chloral hydrate and bromal hydrate: pharmacological contrast and its chemical basis, 401
- Chlorinated tertiary amines, Neurological syndrome induced by administration of some, 249
- Chlorguanide and sulfadiazine, Synergistic effect of, against *Plasmodium gallinaceum* in chick, 60
- Cholinesterase inhibiting activity, Relationship of, to toxicity of some organic phosphorus compounds, 215
- of rat brain, Inhibition of, by methadon, 19
- Citric acid and its sodium salts, Comparative toxic effects of, 65
- Collins, Dean A. Influence of tetraethylammonium on responses of isolated intestine to angiotonin and other substances, 244
- Coronary dilator drugs, Antifibrillatory action of, 136
- Cranston, Elizabeth M., Gisvold, Ole, and Bope, Frank W. Tannin obtained from *Pinus Caribaea*, Morelet, 209
- Cyclopropane anesthesia, Role of arterial pressure in induction of idioventricular rhythms under, 319
- Dauben, William G., Heidelberger, Charles, Heymann, Hans, Seligman, Arnold M., Fieser, Louis F., and Chang, Frederic C. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Davison, Clarke, Smith, Paul K., and Gimble, Abraham I. Toxicity, distribution and excretion of emetine, 431
- Dayrit, Conrado, Kramer, Milton L., Zahm, William, Otto, Harold L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., and Shane, Samuel J. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Shane, Samuel J., Zahm, William, Kramer, Milton L., Gold, Harry, Modell, Walter, and Kwit, Nathaniel T. Thevetin, a glycoside with unusual speed of action in man, 44

- Dearborn, Earl H. Comparison of one dose per day with six doses per day of quinine in suppression of lophuriae malaria in duck, 178  
 Diethylaminoethanol and procaine, Methods for estimation of, 359  
 Digitoxin and histamine, Study of pharmacological relationship between, 53  
 Diphenhydramine and aminophylline, Protective action of combinations of, in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367  
 Eadie, George S., Bernheim, Frederick, and Fitzgerald, Dorothea B. Inhibition of cholinesterase of rat brain by methadon, 19  
 Ellis, Charles H., and Alles, Gordon A. Comparative actions of certain compounds like alpha-fagarine, 416  
 Ellis, Sydney. Enzymic hydrolysis of morphine esters, 130  
 Emetine, Toxicity, distribution and excretion of, 431  
 Enzymic hydrolysis of morphine esters, 130  
 Epinephrine and histamine, antagonism with N-(2-haloalkyl)-1-naphthalene-methylamine derivatives, 339  
 and histamine, Influence of lung distention on response of bronchiles to, 182  
 nicotine and adrenergic nervous reflexes, pressor responses to, Effect of two halogenated ethylamines on, 350  
 Epstein, Herbert J., Krementz, Christie S., and Cahen, Raymond L. Evaluation of analgesic action of methadon isomers and other analgesics by new rapid screening method, 328  
 Ethylamines, Effect of two halogenated, on pressor responses to epinephrine, nicotine and adrenergic nervous reflexes, 350  
 Ferguson, J. K. W., Hamilton, H. S., and Westfall, B. A. Comparison of nine local anesthetics, 299  
 Fibrillation, ventricular, hydrocarbon-epinephrine induced, Studies on, 12  
 Fieser, Louis F., and Heymann, Hans. Naphthoquinone antimalarials. XIX. Antirespiratory study of protein binding, 97  
 Chang, Frederic C., Dauben, William G., Heidelberger, Charles, Heymann, Hans, and Seligman, Arnold M. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85  
 Heymann, Hans, and Seligman, Arnold M. Naphthoquinone antimalarials. XX. Metabolic degradation, 112  
 Fink, Lewis D., and Wright, Harold N. Binding of mapharsen by red blood cell of rabbit *in vitro*, 455, 462  
 Finkelstein, Murray, and Bodansky, Oscar. Effect of cardiac glycosides on respiration of cardiac muscle, 274  
 Fitzgerald, Dorothea B., Eadie, George S., and Bernheim, Frederick. Inhibition of cholinesterase of rat brain by methadon, 19  
 Flinn, Frederick B., and Brodie, Bernard B. Effect on pain threshold of N-acetyl p-aminophenol, 76  
 p-Fluorophenethylamine and p-fluorophenethyl-methylamine, Comparative pharmacology of, 192  
 Frank, Karl, and Wikler, Abraham. Hind-limb reflexes of chronic spinal dogs during cycles of addiction to morphine and methadon, 382  
 Freese, Homer B., Winbury, Martin M., Michiels, Patricia M., and Hamburger, W. E. Protective action of combinations of diphenhydramine and aminophylline in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367  
 Freyburger, Walter A., Moe, Gordon K., Malton, S. Donald, and Rennick, Barbara R. Role of arterial pressure in induction of idioventricular rhythms under cyclopropane anesthesia, 319  
 Garb, Solomon, and Chenoweth, Maynard B. Studies on hydrocarbon-epinephrine induced ventricular fibrillation, 12  
 Gass, S. Richard, Beyer, Karl H., Tillson, Elizabeth K., Russo, Horace F., Schuchardt, Grace S. Absorption and elimination of caronamide, 167  
 Giarman, Nicholas J. Antibiotic lactones and synthetic analogs. I. Cardiotoxic effects on isolated frog heart, 232  
 Gimble, Abraham I., Davison, Clarke, and Smith, Paul K. Toxicity, distribution and excretion of emetine, 431  
 Gisvold, Ole, Bope, Frank W., and Cranston, Elizabeth M. Tannin ob-

- Bronchioles, response of, to epinephrine and histamine, Influence on, of lung distention, 182
- Bronchodilator and antihistamine drugs, Response to, of anaphylactic guinea pig trachea, 412
- Bronchospasm, fatal, in guinea pigs, due to mecholyl aerosol, Protective action of combinations of diphenhydramine and aminophylline in, 367
- Bruce, Margaret D., MacNider, Wm. deB., and Trott, Jesse C., Jr. Inability of BAL to prevent or modify acute renal injury induced by uranium nitrate, 262
- Butler, Thomas C. Bromal hydrate and chloral hydrate: pharmacological contrast and its chemical basis, 401
- Cahen, Raymond L., Epstein, Herbert J., and Krementz, Christie S. Evaluation of analgesic action of methadon isomers and other analgesics by new rapid screening method, 328
- Caplan, H., and Melville, K. I. Influence of lung distention on response of bronchioles to epinephrine and histamine, 182
- Cardiac automaticity, Study of cyclic and noncyclic hydrocarbons on, 315  
muscle, respiration of, Effect on, of cardiac glycosides, 274
- Cardiotoxic effects on isolated frog heart, of antibiotic lactones and synthetic analogs, 232
- Caronamide, Absorption and elimination of, 167
- Carr, C. Jelleff, Vitcha, James F., and Krantz, John C., Jr. Anesthesia. XXXI. Study of cyclic and noncyclic hydrocarbons on cardiac automaticity, 315
- Castillo, Julio C. Anaphylactic guinea pig trachea and its response to antihistamine and bronchodilator drugs, 412
- Central nervous system depressants and local anesthetics, Experimental heart failure caused by, 439
- Chang, Frederic C., Dauben, William G., Heidelberger, Charles, Heymann, Hans, Seligman, Arnold M., and Fieser, Louis F. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Chenoweth, Maynard B., and Garb, Solomon. Studies on hydrocarbon-epinephrine induced ventricular fibrillation, 12
- Chloral hydrate and bromal hydrate: pharmacological contrast and its chemical basis, 401
- Chlorinated tertiary amines, Neurological syndrome induced by administration of some, 249
- Chlorguanide and sulfadiazine, Synergistic effect of, against *Plasmodium gallinaceum* in chick, 60
- Cholinesterase inhibiting activity, Relationship of, to toxicity of some organic phosphorus compounds, 215  
of rat brain, Inhibition of, by methadon, 19
- Citric acid and its sodium salts, Comparative toxic effects of, 65
- Collins, Dean A. Influence of tetraethylammonium on responses of isolated intestine to angiotonin and other substances, 244
- Coronary dilator drugs, Antifibrillatory action of, 136
- Cranston, Elizabeth M., Gisvold, Ole, and Bope, Frank W. Tannin obtained from *Pinus Caribaea*, Morelet, 209
- Cyclopropane anesthesia, Role of arterial pressure in induction of idioventricular rhythms under, 319
- Dauben, William G., Heidelberger, Charles, Heymann, Hans, Seligman, Arnold M., Fieser, Louis F., and Chang, Frederic C. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Davison, Clarke, Smith, Paul K., and Gimble, Abraham I. Toxicity, distribution and excretion of emetine, 431
- Dayrit, Conrado, Kramer, Milton L., Zahm, William, Otto, Harold L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., and Shane, Samuel J. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Shane, Samuel J., Zahm, William, Kramer, Milton L., Gold, Harry, Modell, Walter, and Kwit, Nathaniel T. Thevetin, a glycoside with unusual speed of action in man, 44

- effect of chlorguanide and sulfadiazine against *Plasmodium gallinaceum* in chick, 60
- Karel, Leonard, Jones, H. Walter, Jr., and Meyer, Bertram J. Relationship of cholinesterase inhibiting activity to toxicity of some organic phosphorus compounds, 215
- Kramer, Milton L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Dayrit, Conrado, Shane, Samuel J., and Zahm, William. Thevetin, a glycoside with unusual speed of action in man, 44
- Zahm, William, Otto, Harold L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Shane, Samuel J., and Dayrit, Conrado. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Krantz, John C., Jr., Carr, C. Jelleff, and Vitcha, James F. Anesthesia. XXXI. Study of cyclic and noncyclic hydrocarbons on cardiac automaticity, 315
- Krayer, Otto, and Wollenberger, Albert. Experimental heart failure caused by central nervous system depressants and local anesthetics, 439
- Krementz, Christie S., Cahen, Raymond L., and Epstein, Herbert J. Evaluation of analgesic action of methadon isomers and other analgesics by new rapid screening method, 328
- Kueter, K. E., and Richards, R. K. Antagonism of rutin to capillary effects of sodium bisulfite and procaine, 372
- Kuizenga, Marvin H., and Graham, Boyd E. Pharmacologic study of ortho-methoxy- $\beta$ -phenylisopropyl methylamine hydrochloride, and 13 related methoxy analogues, 150
- Vander Brook, Milton J., Olson, Kenneth J., and Richmond, Marilyn T. Pharmacology of new antihistamine, pyrrolazote, 197
- Kun, E., and Sjoerdsma, A. Study of pharmacological relationship between digoxin and histamine, 53
- Kwit, Nathaniel T., Dayrit, Conrado, Shane, Samuel J., Zahm, William, Kramer, Milton L., Gold, Harry, and Modell, Walter. Thevetin, a glycoside with unusual speed of action in man, 44
- Shane, Samuel J., Dayrit, Conrado, Kramer, Milton L., Zahm, William, Otto, Harold L., Gold, Harry, and Modell, Walter. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Lactones, Antibiotic, and synthetic analogs. I. Cardiotoxic effects on isolated frog heart, 232
- Landing, B. H., Shapiro, D. M., Goldberg, B., Goldin, A., and Noe, H. A. Neurological syndrome induced by administration of some chlorinated tertiary amines, 249
- Laxative activity of triphenylmethane derivatives, 288
- Leblond, C. P., and Grad, B. Thyroxine-like activity of chloro- and bromothyronine derivatives, 125
- Lief, Philip A., Poet, Raymond, and Brodie, Bernard B. Fate of procaine in man following its intravenous administration and methods for estimation of procaine and diethylaminoethanol, 359
- Loew, Earl R., and Micetich, Audrey. Antagonism of histamine and epinephrine with N-(2-haloalkyl)-1-naphthalenemethylaniline derivatives, 339
- and Stone, Clem A. Effects of two halogenated ethylamines on pressor responses to epinephrine, nicotine and adrenergic nervous reflexes, 350
- Loewe, S. Laxative activity of triphenylmethane derivatives. I. Relationship between structure and activity of phenolphthalein congeners, 288
- Lung distention, Influence of, on response of bronchioles to epinephrine and histamine, 182
- MacNider, Wm. deB., Trott, Jesse C., Jr., and Bruce, Margaret D. Inability of BAL to prevent or modify acute renal injury induced by uranium nitrate, 262
- Malaria, lophurae, in duck, suppression of, Comparison of one dose per day with six doses per day of quinine, 178
- Malton, S. Donald, Rennick, Barbara R., Freyburger, Walter A., and Moe, Gordon K. Role of arterial pressure in induction of idioventricular rhythms under cyclopropane anesthesia, 319

- tained from *Pinus Caribaea*, Morelet, 209
- Glycosides, cardiac, Effect of, on respiration of cardiac muscle, 274
- Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Shane, Samuel J., Dayrit, Conrado, Kramer, Milton L., Zahm, William, and Otto, Harold L. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Golbaum, Leo R. Ultraviolet spectrophotometric procedure for determination of barbiturates, 68
- Goldberg, B., Goldin, A., Noe, H. A., Landing, B. H., and Shapiro, D. M. Neurological syndrome induced by administration of some chlorinated tertiary amines, 249
- Goldin, A., Noe, H. A., Landing, B. H., Shapiro, D. M., and Goldberg, B. Neurological syndrome induced by administration of some chlorinated tertiary amines, 249
- Grad, B., and Leblond, C. P. Thyroxine-like activity of chloro- and bromothyronine derivatives, 125
- Graham, Boyd E., and Kuizenga, Marvin H. Pharmacologic study of orthomethoxy- $\beta$ -phenylisopropyl methylamine hydrochloride, and 13 related methoxy analogues, 150
- Green, Ray E., and Wescoe, W. Clarke Mechanism of convulsant action of strychnine; lack of atropine antagonism, 78
- Greenberg, Joseph, Boyd, Beverly L., and Josephson, Edward S. Synergistic effect of chlorguanide and sulfadiazine against *Plasmodium gallinaceum* in chick, 60
- Gruber, Charles M., Jr., and Halbeisen, William A. Comparative toxic effects of citric acid and its sodium salts, 65
- Halbeisen, William A., and Gruber, Charles M., Jr. Comparative toxic effects of citric acid and its sodium salts, 65
- Hambourger, W. E., Freese, Homer B., Winbury, Martin M., and Michiels, Patricia M. Protective action of combinations of diphenhydramine and aminophylline in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367
- Hamilton, H. S., Westfall, B. A., and Ferguson, J. K. W. Comparison of nine local anesthetics, 299
- Heart failure, Experimental, caused by central nervous system depressants and local anesthetics, 439
- muscle, respiration of, Action on, of narcotics and local anesthetics, 444
- Heidelberger, Charles, Heymann, Hans, Seligman, Arnold M., Fieser, Louis F., Chang, Frederic C., and Dauben, William G. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Heptylamines, isomeric, Comparative pharmacology of, 225
- Heymann, Hans, Seligman, Arnold M., and Fieser, Louis F. Naphthoquinone antimalarials. XX. Metabolic degradation, 112
- and Fieser, Louis F. Naphthoquinone antimalarials. XIX. Antirespiratory study of protein binding, 97
- Seligman, Arnold M., Fieser, Louis F., Chang, Frederic C., Dauben, William G., and Heidelberger, Charles. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- Histamine and digitoxin, Study of pharmacological relationship between, 53
- and epinephrine, antagonism with N-(2-haloalkyl)-1-naphthalenemethylamine derivatives, 339
- and epinephrine, Influence of lung distention on response of bronchioles to, 182
- Hutcheon, D. E. Biological assay of thyroid activity, 303
- Hydrocarbon-epinephrine induced ventricular fibrillation, Studies on, 12
- Hydrocarbons, cyclic and noncyclic, Study of, cardiac automaticity, 315
- Jones, H. Walter, Jr., Meyer, Bertram J., and Karel, Leonard. Relationship of cholinesterase inhibiting activity to toxicity of some organic phosphorus compounds, 215
- Josephson, Edward S., Greenberg, Joseph, and Boyd, Beverly L. Synergistic

- Pain threshold, effect on, of N-acetyl p-aminophenol, 76
- Phenolphthalein congeners, Relationship between structure and activity, 288
- 1-Phenyl-2-butylamine, Pharmacological activity of, 426
- Phosphorus, organic, compounds, Relationship of cholinesterase inhibiting activity to toxicity of, 215
- Pinus Caribaea*, Morelet, Tannin obtained from, 209
- Plasmodium gallinaceum* in chick, Synergistic effect against, of chlorguanide and sulfadiazine, 60
- Poet, Raymond, Brodie's Bernard B., and Lief, Philip A. Fate of procaine in man following its intravenous administration and methods for estimation of procaine and diethylaminoethanol, 359
- Procaine, Fate of, in man, following its intravenous administration and methods for estimation of procaine and diethylaminoethanol, 359
- Procaine and sodium bisulfite, Antagonism of rutin to capillary effects of, 372
- Pyrrolazote, new antihistamine, Pharmacology of, 197
- Quinine, Comparison of one dose per day with six doses per day, in suppression of lophurae malaria in duck, 178
- Renal injury, acute, induced by uranium nitrate, Inability of BAL to prevent or modify, 262
- Rennick, Barbara R., Freyburger, Walter A., Moe, Gordon K., and Malton, S. Donald. Role of arterial pressure in induction of idioventricular rhythms under cyclopropane anesthesia, 319
- Richards, R. K., and Kueker, K. E. Antagonism of rutin to capillary effects of sodium bisulfite and procaine, 372
- Richmond, Marilyn T., Kuizenga, Marvin H., Vander Brook, Milton J., and Olson, Kenneth J. Pharmacology of new antihistamine, pyrrolazote, 197
- Russo, Horace F., Schuchardt, Grace S., Gass, S. Richard, Beyer, Karl H., and Tillson, Elizabeth K. Absorption and elimination of caronamide, 167
- Rutin, Antagonism of, to capillary Effects of sodium bisulfite and procaine, 272
- Schuchardt, Grace S., Gass, S. Richard, Beyer, Karl H., Tillson, Elizabeth K., and Russo, Horace F. Absorption and elimination of caronamide, 167
- Schubert, Jack, and McClinton, L. T. Toxicity of some zirconium and thorium salts in rats, 1
- Seligman, Arnold M., Fieser, Louis F., and Heymann, Hans. Naphthoquinone antimalarials. XX. Metabolic degradation, 112
- Fieser, Louis F., Chang, Frederic C., Dauben, William G., Heidelberger, Charles, and Heymann, Hans. Naphthoquinone antimalarials. XVIII. metabolic oxidation products, 85†
- Shane, Samuel J., Dayrit, Conrado, Kramer, Milton L., Zahm, William, Otto, Harold L., Gold, Harry, Modell, Walter, and Kwit, Nathaniel T. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Zahm, William, Kramer, Milton L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., and Dayrit, Conrado. Thevetin, a glycoside with unusual speed of action in man, 44
- Shapiro, D. M., Goldberg, B., Goldin, A., Noe, H. A., and Landing, B. H. Neurological syndrome induced by administration of some chlorinated tertiary amines, 249
- Sjoendsma, A., and Kun E. Study of pharmacological relationship between digoxin and histamine, 53
- Smith, Paul K., Gimble, Abraham I., and Davison, Clarke. Toxicity, distribution and excretion of emetine, 431
- Sodium bisulfite and procaine, Antagonism of rutin to capillary effects of, 372
- Stone, Clem A., and Loew, Earl R. Effects of two halogenated ethylamine on pressor responses to epinephrine, nicotine and adrenergic nervous reflexes, 350
- Strophanthidin-3-acetate, Comparison of, with ouabain by intravenous injection in man, 39
- Strychnine, Mechanism of convulsant action of, lack of atropine antagonism, 78
- Sulfadiazine and chlorguanide, Synergistic effect of, against *Plasmodium gallinaceum* in chick, 60

- Mapharsen, Binding of, by red blood cell of rabbit *in vitro*, 455, 462
- Marsh, David F. Comparative pharmacology of isomeric heptylamines, 225
- Comparative pharmacology of p-fluorophenethylamine and p-fluorophenethylmethylamine, 192
- Pharmacological activity of 1-phenyl-2-butylamine, 426
- and Van Liere, Edward J. Effect of adrenergic blocking agents on vasoconstriction produced by acute oxygen lack, 221
- McClinton, L. T., and Schubert, Jack. Toxicity of some zirconium and thorium salts in rats, 1
- Melville, K. I. Antifibrillatory action of coronary dilator drugs, 136
- and Caplan, H. Influence of lung distention on response of bronchioles to epinephrine and histamine, 182
- Methadon and morphine, Hindlimb reflexes of chronic spinal dogs during cycles of addiction to, 382
- Inhibition by, of cholinesterase of rat brain, 19
- isomers and other analgesics, Evaluation of analgesic action of, by new rapid screening method, 328
- Meyer, Bertram J., Karel, Leonard, and Jones, H. Walter, Jr. Relationship of cholinesterase inhibiting activity to toxicity of some organic phosphorus compounds, 215
- Micetich, Audrey, and Loew, Earl R. Antagonism of histamine and epinephrine with N-(2-haloalkyl)-1-naphthalenemethylamine derivatives, 339
- Michiels, Patricia M., Hambourger, W. W., Freese, Homer B., and Winbury, Martin M. Protective action of combinations of diphenhydramine and amino-phylline in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 367
- Modell, Walter, Kwit, Nathaniel T., Dayrit, Conrado, Shane, Samuel J., Zahm, William, Kramer, Milton L., and Gold, Harry. The vetin, a glycoside with unusual speed of action in man, 44
- Kwit, Nathaniel T., Shane, Samuel J., Dayrit, Conrado, Kramer, Milton L., Zahm, William, Otto, Harold L., and Gold, Harry. Comparison of ouabain with strophantidin-3-acetate by intravenous injection in man, 39
- Moe, Gordon K., Malton, S. Donald, Renick, Barbara R., and Freyburger, Walter A. Role of arterial pressure in induction of idioventricular rhythms under cyclopropane anesthesia, 319
- Morphine and methadon, Hindlimb reflexes of chronic spinal dogs during cycles of addiction to, 382
- esters, Enzymic hydrolysis of, 130
- N-acetyl p-aminophenol, Effect of, on pain threshold, 76
- Naphthoquinone antimalarials. XVIII. Metabolic oxidation products, 85
- XIX. Antirespiratory study of protein binding, 97
- XX. Metabolic degradation, 112
- Narcotics and local anesthetics, Action of, on respiration of heart muscle, 444
- Nicotine, epinephrine and adrenergic nervous reflexes, pressor responses to, Effect of two halogenated ethylamines on, 350
- Noe, H. A., Landing, B. H., Shapiro, D. M., Goldberg, B., and Goldin, A. Neurological syndrome induced by administration of some chlorinated tertiary amines, 249
- Olson, Kenneth J., Richmond, Marilyn T., Kuizenga, Marvin H., and Vander Brook, Milton J. Pharmacology of new antihistamine, pyrrolazote, 197
- Ortho-methoxy- $\beta$ -phenylisopropyl methylamine hydrochloride, Pharmacologic study of, and 13 related methoxy analogues, 150
- Otto, Harold L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Shane, Samuel J., Dayrit, Conrado, Kramer, Milton L., and Zahm, William. Comparison of ouabain with strophantidin-3-acetate by intravenous injection in man, 39
- Ouabain, Comparison of, with strophantidin-3-acetate by intravenous injection in man, 39
- Oxygen lack, acute, vasoconstriction produced by, Effect of adrenergic blocking agents on, 221



- Tannin obtained from *Pinus Caribaea*, Morelet, 209
- Tetraethylammonium, Influence of, on responses of isolated intestine to angiotonin and other substances, 244
- Thevetin, a glycoside with unusual speed of action in man, 44
- Thorium and zirconium salts, Toxicity of, in rats, 1
- Thyroid activity, Biological assay of, 303
- Thyronine derivatives (chloro- and bromo-), Thyroxine-like activity of, 125
- Tillson, Elizabeth K., Russo, Horace F., Schuchardt, Grace S., Gass, S. Richard, and Beyer, Karl H. Absorption and elimination of caronamide, 167
- Trachea, guinea pig, Anaphylactic, Response of, to antihistamine and bronchodilator drugs, 412
- Triphenylmethane derivatives, Laxative activity of, 288
- Trott, Jesse C., Jr., Bruce, Margaret D., and MacNider, Wm. deB. Inability of BAL to prevent or modify acute renal injury induced by uranium nitrate, 262
- Uranium nitrate, acute renal injury induced by, Inability of BAL to prevent or modify, 262
- Van Liere, Edward J., and Marsh, David F. Effect of adrenergic blocking agents on vasoconstriction produced by acute oxygen lack, 221
- Vander Brook, Milton J., Olson, Kenneth J., Richmond, Marilyn T., and Kui-zenga, Marvin H. Pharmacology of new antihistamine, pyrrolazote, 197
- Vasoconstriction produced by acute oxygen lack, Effect on, of adrenergic blocking agents, 221
- Vitcha, James F., Krantz, John C., Jr., and Carr, C. Jelleff. Anesthesia. XXXI. Study of cyclic and noncyclic hydrocarbons on cardiac automaticity, 315
- Wescoc, W. Clarke, and Green, Ray Mechanism of convulsant action strychnine; lack of atropine antagonism, 78
- Westfall, B. A., Ferguson, J. K. W., and Hamilton, H. S. Comparison of local anesthetics, 299
- Wikler, Abraham, and Frank, Karl. Hind limb reflexes of chronic spinal dogs during cycles of addiction to morphine and methadon, 382
- Winbury, Martin M., Michiels, Patri M., Hamburger, W. E., and Free Homer B. Protective action of combinations of diphenhydramine and aminophylline in fatal bronchospasm in guinea pigs due to mecholyl aerosol, 1
- Winter, Charles A. Potentiating effect of antihistaminic drugs on sedative action of barbiturates, 7
- Wollenberger, Albert. Action of narcotics and local anesthetics on respiration and heart muscle, 444
- and Krayer, Otto. Experimental heart failure caused by central nervous system depressants and local anesthetics, 439
- Wright, Harold N., and Fink, Lewis. Binding of mapharsen by red blood cells of rabbit, *in vitro*, 455, 462
- Zahm, William, Kramer, Milton L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Dayrit, Conrado, and Samuel J. Thevetin, a glycoside with unusual speed of action in man, 44
- Otto, Harold L., Gold, Harry, Modell, Walter, Kwit, Nathaniel T., Samuel J., Dayrit, Conrado, and Kramer, Milton L. Comparison of ouabain with strophanthidin-3-acetate by intravenous injection in man, 39
- Zirconium and thorium salts, Toxicity in rats, 1

